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14. ABSTRACT
Animal research suggests that reactivation (retrieval) of a consolidated memory can return it to a labile state from which it must be restabilized in order to persist. This stabilization process has been termed "reconsolidation", and various behavioral and pharmacological interventions have been found to modify or block it. The aim of this project was to create an experimental assay in the form of an optimal Pavlovian differential fear-conditioning paradigm, within which the relative strengths of various pharmacological and behavioral, reconsolidation-blocking interventions could be tested. We completed testing for two pharmacological interventions and a behavioral intervention. Study of a third pharmacological intervention was initiated. Results from propranolol and behavioral intervention groups demonstrated differential conditioning learning on Day 1, supporting the validity of our modified fear-conditioning paradigm. Propranolol administration and the behavioral intervention at the time of memory reactivation did not decrease the fear memory, as indexed by skin conductance, when assessing renewal and reinstatement. Mifepristone was tested as a second pharmacological intervention. After adjusting for initial differences in conditioned response strength, results suggest that mifepristone did reduce the fear memory. Results from a third pharmacological intervention, oxytocin, tentatively suggest a generalized reduction of the fear memory.

15. SUBJECT TERMS
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1. INTRODUCTION

Background: Animal research suggests that reactivation (retrieval) of a consolidated memory can return it to a labile state from which it must be restabilized in order to persist. This stabilization process has been termed "reconsolidation," and various pharmacological and non-pharmacological interventions can block it. This ability offers novel therapeutic possibilities for PTSD. To date, few human studies have been conducted on the mechanism of memory reconsolidation blockade; some of them have yielded positive results but their clinical relevance to the problem of PTSD remains very limited. Two recent studies performed in healthy human subjects have addressed the question of memory reconsolidation blockade using fear-conditioning paradigms, which are highly relevant to PTSD. Both studies demonstrated that fear memory could be eliminated via the mechanism of reconsolidation. The first study (Kindt et al. 2009) demonstrated the phenomenon using a beta-blocker, propranolol, whereas the other study (Schiller et al. 2010) obtained similar results using a non-pharmacological/behavioral intervention that combines an extinction protocol within a reconsolidation paradigm (delayed extinction). Given that both studies quickly and completely abolished fear responses, this floor effect prevents us from using their study design in order to test the relative strengths of various reconsolidation-blocking (or memory updating) interventions. In other words, if propranolol or delayed extinction totally eliminates the conditioned fear response, no other intervention could be found to be superior.

Goals: The specific aim of the present project is to create an experimental assay in the form of an optimal Pavlovian differential fear conditioning paradigm within which the relative strengths of various novel behavioral and pharmacological reconsolidation-based interventions can be compared. In order to accomplish this, we designed a new experimental protocol that is free of floor effects. Specifically, we tested the following modifications to existing experimental designs: 1) use of a more highly "prepared" (i.e., danger-signaling) conditioned stimulus (CS); 2) recruitment of more sensitive subjects; 3) selection of only subjects who acquire strong conditioned responses (CRs) during conditioning for further participation, and 4) use of additional probes for the presence of the latent CR, viz., renewal and savings in addition to spontaneous recovery and reinstatement.

General procedure: The protocol consisted of four distinct visits that took place over the course of a month. On Day 1 (habituation and acquisition), subjects viewed video clips of three rooms (contexts) different in color and content, presented on a 42" high definition television. The stimuli were three different videos of tarantulas, one presented in the context of each room. Two of the three tarantulas served as the two CS+s and the third as the CS-. Each CS+ presentation was sometimes followed by shock (i.e. reinforced); the CS- was never be followed by shock. During this first session, each CS was presented twice in an unreinforced manner (habituation phase). Next, during the acquisition phase, there were 8 presentations of each CS, with 5 of each CS+ presentations followed by shock (i.e. 62.5% reinforcement). The acquisition phase took place in Context A. On Day 2 (intervention), subjects were assigned to one of four interventions: pharmacological (propranolol, mifepristone, oxytocin) or behavioral. For the pharmacological interventions, participants received: 1) 40 mg of propranolol (oral administration) or 2) 1800 mg of mifepristone (oral administration) followed 90 minutes later by a single, unreinforced presentation of one of the two CS+, designated the reactivated CS+ (CS+R), or 3) 32 IU of

oxytocin (intranasal administration) followed 30 minutes later by a single, unreinforced presentation of CS+R. For the behavioral intervention, participants were exposed to a single, unreinforced presentation of the CS+R without receiving a drug, followed 10 minutes later by 10 further unreinforced CS+R presentations, 11 unreinforced presentations of the remaining CS+ (designated the CS+ with no intervention – CS+N) and 11 CS- presentations. All presentations related to Day 2 took place in context B. Day 3 was divided into two different components, all took place in context A. First, each CS (CS+R, CS+N and CS-) was presented twice in an unreinforced manner in order to assess renewal. This was followed by the presentation of 3 shocks alone and 8 further unreinforced presentations of each CS (CS+R, CS+N and CS-) in order to evaluate reinstatement. Finally, on Day 30, all presentations took place in a new context, namely context C. In order to evaluate spontaneous recovery, 8 unreinforced presentations of each CS (CS+R, CS+N, CS-) were presented first. This was followed by 8 presentations of all CSs, with the two CS+s being reinforced 62.5% of the time. This second acquisition phase allowed us to examine savings during re-acquisition.

Importantly, although the same spider is always used for the CS-, two different task versions were designed so that the two remaining spiders are alternated regarding whether they serve as CS+R or CS+N.

2. BODY
STUDY 1: PROPRANOLOL INTERVENTION

Pre-Reactivation Propranolol Fails to Reduce Skin Conductance Reactivity to Prepared Fear-Conditioned Stimuli

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Abstract

Pharmacologic blockade of memory reconsolidation has been demonstrated in fear-conditioned rodents and humans and may provide a means to reduce fearfulness in anxiety disorders and posttraumatic stress disorder. Studying the efficacy of potential interventions in clinical populations is challenging, creating a need for paradigms within which candidate reconsolidation blocking interventions can be readily tested. We used videos of biologically prepared, conditioned stimuli (tarantulas) to test the efficacy of propranolol in blocking reconsolidation of conditioned fear in healthy young adults. Strong differential conditioning, measured by skin conductance, was observed among a screened subset of participants during acquisition. However, subsequent propranolol failed to reduce reactivity to the reactivated conditioned stimulus. These results are consistent with other recent findings and point to a need for testing other candidate drugs.

Pre-Reactivation Propranolol Fails to Reduce Skin Conductance Reactivity to Prepared Fear-Conditioned Stimuli

Once formed, a fear memory must stabilize if it is to persist. This process, termed consolidation, occurs within a narrow window, i.e., minutes to hours, during which the memory is labile and susceptible to intervention (Dudai, 2004; Walker, Brakefield, Hobson, & Stickgold, 2003). Potential clinical opportunities arise from this consolidation window, including interventions for posttraumatic stress disorder (PTSD). Stress hormones may potentiate consolidation and thereby produce a memory trace that is easily activated and resistant to extinction. This process may be involved in the pathogenesis of PTSD (Pitman, 1989). Pharmacological agents, including beta-adrenergic antagonists, could limit the memory-modulating effects of these hormones (McGaugh, 2004) and in so doing attenuate excessive consolidation, if administered during the window. However, this approach is complicated by the need to intervene before the memory has consolidated. Studies attempting to do this have produced mixed results (Hoge et al., 2012; Holmes, James, Coode-Bate, & Deeprose, 2009; Krauseneck et al., 2010; Nugent et al., 2010; Pitman et al., 2002; Stein, Kerridge, Dimsdale, & Hoyt, 2007; Vaiva et al., 2003).

As demonstrated in animal research, reactivation (i.e., retrieval) of a consolidated memory returns it to a destabilized state, from which it must be restabilized (i.e. reconsolidated) if it is to persist (Debiec & Ledoux, 2004; Nader, Schafe, & Le Doux, 2000; Nader & Einarsson, 2010). Reconsolidation is governed by neurobiological processes similar to those of consolidation (Lee, Everitt, & Thomas, 2004), and is also susceptible to pharmacological blockade at the level of stress hormone receptors (Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Pitman et al., 2011; Przybyslawski, Roullet, & Sara, 1999). Because reactivation of trauma memories can be planned in advance, but traumatic events cannot, interference with memory *reconsolidation* may offer a more feasible clinical target. Brunet and colleagues have extended the above reconsolidation findings to individuals with PTSD (Brunet et al., 2008). Within a double-blind randomized control trial, those participants who received propranolol prior to recalling their traumatic memory exhibited significantly lower overall physiological reactivity during a subsequent laboratory visit when they again recalled their traumatic experience, suggesting that the traumatic memory, or at least its emotional component, had been weakened.

Although clinical application remains the ultimate goal, there persists the need for a basic paradigm wherein candidate pharmacological agents can be more readily tested. Relatively few studies have investigated reconsolidation blockade in humans, and fewer still have done so in a normal (i.e., non-PTSD) population. Recent studies performed in healthy human subjects have helped to address this gap (Kindt, Soeter, & Vervliet, 2009; Soeter & Kindt, 2010, 2011). Soeter & Kindt (2010) used pictures of spiders as a fear-relevant conditioned stimulus (CS) in a differential fear conditioning paradigm with potentiated eyeblink startle response serving as the conditioned response (CR). After first learning to associate one spider (CS+), but not another spider (CS-), with shock, participants received either propranolol or placebo in conjunction with a single reactivation trial of the CS+ alone in the absence of shock. A third group of subjects received propranolol without memory reactivation of the CS+. Finally, subjects viewed ten unreinforced presentations each of the CS+ and CS-. Participants in the placebo-reactivation and propranolol-non-reactivation groups showed strong eyeblink responses to the CS+, compared to

the CS-, consistent with preservation of the conditioned fear memory. In contrast, subjects in the propranolol-reactivation group showed comparably small eyeblink responses to the CS+ and CS-, suggesting erasure of the fear memory. Furthermore, the eyeblink response remained small in the propranolol-reactivation group in a subsequent reinstatement test. The investigators interpreted their findings as indicating blockade of reconsolidation of the conditioned fear response following memory reactivation by presentation of the CS+ accompanied by propranolol.

The findings of Soeter and Kindt (2010) suggest that: a) reconsolidation and its pharmacological blockade generalize to a non-clinical model of fear memory in humans, and b) propranolol is efficacious in the abolishment of conditioned fear as measured by eyeblink startle response. However, the protocol also produced a floor effect, wherein there was a 100% reduction of the fear memory trace in the propranolol-reactivation condition. This floor effect precludes comparison of the relative strengths of various candidate interventions; an intervention that is potentially more effective than propranolol at reducing memory reconsolidation could not produce more than 100% reduction in this paradigm. In an attempt to overcome this limitation and build upon the collective findings of Kindt and Soeter, we sought to create and validate an optimal Pavlovian fear conditioning paradigm that could be used to test the relative strengths of various drug and non-drug candidates for reconsolidation blockade. The paradigm we sought to create needed to be more resistant to total blockade, i.e., produce significant but only partial (i.e., <100%) reduction of the fear memory. To this end, we employed more highly prepared CSs, more fear-sensitive subjects, and stronger conditioned responses (CRs).

It has been shown that certain classes of CSs, when paired with a US, produce a stronger fear CR, i.e., they are more "prepared" to enter into an association with the US (Mineka & Öhman, 2002). Kindt and colleagues (Kindt et al., 2009; Soeter & Kindt, 2010) used prepared CSs, viz., still pictures of spiders. We enhanced preparedness of the CSs by using 12-sec, high-definition video clips of three crawling tarantulas, each conspicuously different in appearance. Second, we limited enrollment to participants who scored approximately one standard deviation or greater above the population mean on the Spider Phobia Questionnaire-15 (SPQ-15), as described by Olatunji et al. (2009). However, we did exclude anyone who endorsed symptoms of clinical spider phobia (see Methods). Third, we required that participants show evidence of strong differential conditioning, as determined by a more stringent cutoff assigned to the CRs recorded during Day 1 acquisition (specified below). Participants with subthreshold CRs were withdrawn after Day 1. Soeter and Kindt (2010) applied a less stringent cutoff (i.e., mean acquisition trials 7-8 CS+ > CS-).

We also aimed to test the efficacy of propranolol in blocking reconsolidation and reducing fear memory within this paradigm, as measured by changes in skin conductance (SC). Soeter and Kindt (2010) recorded both eyeblink startle and SC responses (SCRs) and, while total abolishment of the differential potentiated eyeblink response was observed, there was no reduction by propranolol of the conditioned SCR. Failure of the SC response to support reconsolidation blockade is a bit perplexing, given that this has been a widely used measure of human fear conditioning (see Boucsein, 2012). Moreover, it is unclear what impact the presentation of noise stimuli (required to elicit the eyeblink response) during the CS interval may have had on SCR in the Soeter and Kindt paradigm, as these startle probes introduce an additional US (i.e. a 104 dB loud noise). In order to avoid the potential confound produced by the startle probes, we chose to only record SC responses.

Method

Participants

Prior to enrollment, participants were screened by phone to verify a) absence of medical conditions that would contraindicate administration of propranolol, e.g., asthma, hypotension, diabetes, and b) presence of a manageable, non-phobic fear of spiders as determined by scores above the mean on the SPQ-15 (Olatunji et al., 2009) and phobia criteria extracted from the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-IV; First, Spitzer, Gibbon, & Williams, 1997). Participants underwent a set of screening criteria taken directly from the SCID-IV to verify absence of current psychiatric disorders, serious medical or neurological conditions, brain injury, and current or past substance abuse. A positive response to a screening criterion led to a full examination of that criterion per SCID-IV. A urine drug screen verified the absence of illicit substances and psychotropic medications. The presence of a current Axis I psychiatric disorder or illicit substances/medications was grounds for withdrawal from the study.

Fifty-five healthy participants (35 females, 20 males) were enrolled in the study. Of these, three were withdrawn before commencing the Day-1 procedure due to: unmeasurable (very low) SC levels (n=2), and presence of a current Axis I psychiatric disorder (n=1). An additional 28 were withdrawn after Day 1 due to: non-compliance (n = 1), data collection error (n = 1), drop out (n = 1), and failure to demonstrate adequate differential conditioning (n = 25; conditioning criteria are described below in Data Reduction). The remaining 24 (12F, 12M) who underwent study procedures on Days 1-3 had a mean age of 22.6 years (SD = 3.2, range 18 to 31 years) and a mean score on the SPQ-15 of 8.0 (SD = 1.8, range 6 to 11 of a possible 0 to 15). Mean years of education was 15.5 (SD = 1.9, range 12 to 19); three participants failed to provide this information. Seven of the 24 participants were not included in Day 30 analyses due to: data collection error (n = 1), and being lost to follow-up (n = 6).

The study protocol was approved by the Partners Human Research Committee (PHRC), as well as the United States Army Medical Research and Material Command (USAMRMC) Human Research Protection Office (HRPO). After a full explanation of the procedures, all participants provided written informed consent.

Equipment and Stimuli

Skin conductance analog signals were recorded using a Coulbourn Lab Linc V Series Human Measurement System (Coulbourn Instruments, Whitehall, PA) with a Coulbourn Isolated Skin Conductance Coupler (V71-23) through 8mm (sensor diameter) Ag/AgCl electrodes (In Vivo Metric; Healdsburg, CA) filled with an isotonic paste. Electrodes were separated by 14mm, as determined by the width of the adhesive collar, and placed on the hypothenar surface of the subject's non-dominant hand in accordance with published guidelines (Boucsein et al., 2012; Fowles et al., 1981). The SC signal was sampled at 1000 Hz and digitized by a Coulbourn Analog to Digital Converter (V19-16). A Cobalt notebook computer (IBM-compatible; Cobalt Computers, Whitehall, PA) with custom-designed software was used to record and store the digitized physiological signals.

Conditioned stimuli (CSs) consisted of nine high-definition video clips (Virtually Better Inc., Decatur, GA) depicting one of three tarantulas occupying one of three contexts. Two of the three tarantulas always served as the CS+s, either the to-be-reactivated CS+ (CS+R) or the to-be-non-reactivated CS+ (CS+N), and were paired with the unconditioned stimulus (US, shock) on day 1.

The to-be CS+R served as the stimulus that was presented on day 2 after receiving the study medication; the to-be CS+N was not presented on day 2. The third tarantula served as the CS- and was not paired with the US and not presented on day 2. The three contexts within which the tarantulas appeared were a kitchen (A), bedroom (B) and office (C). The particular tarantula that served as the CS+N or CS+R was counterbalanced across participants; the tarantula used to represent the CS- was the same across subjects.

The US was a 0.5-sec mild electric shock ranged in intensity (0.2 to 4.0 milliamperes) according to the level selected by the participant and determined to be "highly annoying but not painful." The US was delivered using a Coulbourn Transcutaneous Aversive Finger Stimulator (E13-22) through shock electrodes attached to the middle segments of the 2nd and 3rd fingers on the hand opposite to that on which the SC recording electrodes were attached.

Video clips lasted 12 seconds: four seconds of context alone (i.e., no tarantula), followed by eight seconds of context plus tarantula. On reinforced trials, the US immediately followed the CS+. The intertrial interval consisted of a black screen and was randomized to last 16, 18, 20, 22, or 24 seconds. The procedure was implemented using E-Prime Professional 2.0 (Psychology Software Tools, Inc., Sharpsburg, PA).

Procedure

As depicted in Figure 1, the procedure consisted of a differential fear-conditioning paradigm that entailed laboratory visits over three consecutive days and a one-month follow-up visit (M = 29.9 days; SD = 2.7). On day 1, participants were instructed: *"Today, you will be viewing videos of spiders on the television, and you will receive electric shocks on your fingers after viewing some of the spiders. These shocks will be annoying, but not painful. We will also use electrodes on your palm to record how your body responds to this procedure."* Following these instructions, participants set the shock to a level to be "highly annoying but not painful" (Orr et al., 2000). Participants were then shown still images, i.e., screenshots, of the three tarantulas that would serve as CSs, accompanied by these instructions: *"During the experiment, it will be important that you are able to tell these spiders apart. To do this, try focusing on the legs. For this spider, note the alternating black and white stripe pattern. For this spider, note the orange highlights. For this spider, note that the legs are solid black."* Prior to beginning the procedure, the lights were dimmed and over-ear headphones placed on the participant to reduce ambient noise and enable communication with study staff in the next room. Participants were instructed to sit still in the chair, keep their eyes open, and be attentive to the stimuli presented on the screen. Next, there was a 5-min baseline period to record physiological levels.

Day 1 consisted of two sequential phases: 1) unreinforced presentations of each of the nine possible spider-context combinations in pseudorandom order (*habituation*), and 2) eight partially reinforced (i.e., five of eight) presentations each of CS+R and CS+N, presented separately in blocks and interspersed pseudorandomly with eight presentations of CS- (*acquisition*). The order of presentation of CS+R and CS+N trial blocks was counterbalanced across participants. All CS+R, CS+N, and CS- presentations during acquisition occurred within context A. Participants who did not meet the defined cutoff for demonstrating a differential conditioned response (see below) were withdrawn prior to day 2.

The procedures for days 2, 3, and the one-month follow-up were largely the same as for day 1, with the following exceptions: a) the procedure for setting the level of shock was not repeated, as the shock level determined on the first visit was used for the remainder of the study, b) participants were only familiarized with images of the stimuli prior to undergoing the day 1 procedure, and c) rather than "will receive" as on day 1, participants were instructed that they "may or may not receive" electric shocks.

Day 2 consisted of the participant receiving a 40 mg oral dose of short-acting propranolol (Mylan Pharmaceuticals, Pittsburgh, PA) followed 90 minutes later by a single, unreinforced presentation of the CS+R (*reactivation*). Propranolol reaches peak plasma levels approximately 90 minutes after ingestion (Gilman & Goodman, 1996). Reactivation of the CS+R occurred in context B. On day 2, blood pressure was measured immediately prior to administration of propranolol and again immediately following the single presentation of CS+R. Paired t-tests were used to compare the time points and thus verify the expected physiological effects of propranolol. A significant reduction in systolic ($t(19) = 5.44$, $p < .001$), but not diastolic ($t(19) = .68$, $p = .25$) blood pressure was observed. Also, there was a significant decrease in pulse measured at those same time points ($t(19) = 7.04$, $p < .001$).

Day 3 consisted of three sequential phases: 1) two unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*renewal test*); and 2) three unsignalled presentations of the US alone*,* followed by 3) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*reinstatement test trials* and *extinction*). All presentations of the CSs occurred in context A. Ordering of CS+R and CS+N presentations, within the full set of trials that included CS- presentations, was counterbalanced across subjects.

The one-month follow-up consisted of two phases: 1) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*spontaneous recovery test* and *re-extinction*), followed by 2) eight partially-reinforced, i.e., five of eight, presentations each of CS+R and CS+N presented in successive blocks and interspersed with eight CS- trials for the respective blocks as was done on day 1 (*re-acquisition/savings test*). All stimuli were presented in context C during this visit, and the CS+R block of trials was presented first.

Physiological Measures and Data Reduction

As previously described (Milad, Orr, Pitman, & Rauch, 2005; Orr et al., 2000), an SCR for the CS interval was calculated for each trial by subtracting the mean SC level during the two sec prior to CS onset (context alone presentation) from the peak SC level during the eight sec CS interval. These SCR values reflect change in skin conductance level beyond that resulting from presentation of context alone. A square root transformation was applied to the absolute value of each SCR, followed by replacement of the + or - sign, prior to statistical analysis.

For day 1, the untransformed SCR data were scored to determine whether a definable differential SCR was obtained for *both* the CS+R and CS+N during the acquisition phase. Averaging SCRs across respective CS+R, CS+N, and CS- trials, we calculated a difference score between the CS+R and its respective CS- trials and between the CS+N and its respective CS- trials. A cutoff of $.1\mu S$ was applied to each difference score. Participants with one or both difference scores below this cutoff were withdrawn from the study prior to Day 2.

Results

Mixed-model, repeated measures analyses of variance (ANOVA) using the Statistical Analysis System (SAS, 2012) SAS/STAT^{TM} Version 9.3 software PROC MIXED with TYPE = UN specified for the covariance matrix was performed separately for the acquisition (day 1), renewal (day 3), reinstatement (day 3), re-extinction (1 month) and re-acquisition (1 month) phases with Participants as a random effect, Stimulus (CS+R, CS+N, CS-) as a within-participants effect and Trials as a repeated measure.

Acquisition Phase (Day 1).

Responses to the CSs during the acquisition phase were examined across the 8 presentations of each CS using a two-factor (Stimulus, Trials), repeated measures model with the Stimulus factor having 2 levels in each of 3 ANOVAs comparing CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials; the Trials factor has 8 levels. As can be seen in Table 1 and Figure 2, Panel A, during the acquisition phase, the CS+R and CS+N demonstrated comparably larger SCRs, compared to their respective CS- trials. The magnitude of the SCRs did not differ between CS+R and CS+N trials. There was a significant Stimulus x Trial interaction ($F(7, 161) = 2.19$, $p = .04$, $Eta^2 = .01$) when comparing CS+R to CS-, but not when comparing the CS+N to CS- ($F(7, 161) < 1$, p = NS, $Eta^2 = .01$).

SC responses for the US interval, which represented the unconditioned response (UR), were calculated and plotted for the acquisition phase (see Figure 3). Because SCR onset has a known latency of 1-2 s (Edelberg, 1967), the 1-s interval immediately following US onset was used as the baseline for calculating the SC UR, which was subtracted from the peak SC level within the 6-s interval following US onset to yield the UR. A square root transformation was applied to the UR, as was done for the CR. As expected, both CS+R and CS+N trials produced larger SCRs during the US interval, compared to CS- trials ($F(1, 23) = 275.0$, $p < .001$, $Eta^2 = .40$; $F(1, 23) = 237.3$, $p < .001$, $Eta^2 = .46$, respectively). There were significant Stimulus x Trial interactions ($F(7, 161) = 27.5$, $p < .001$, $Eta^2 = .14$; $F(7, 161) = 31.3$, $p < .001$, $Eta^2 = .11$, respectively). The pattern of URs for CS+R and CS+N across reinforced trials was similar, characterized by a large response on the first trial and lower responses for subsequent trials, with a slight increase in responses across the last three trials (Figure 3).

Renewal Phase (Day 3).

Responses to the CSs during the renewal phase were examined across the 2 presentations of each CS using a two-factor (Stimulus, Trials) repeated measures model. The Stimulus factor has two levels in each of three ANOVAs that compared CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials; the Trials factor has 2 levels. As can be seen in Table 1 and Figure 2, Panel B, SCRs to the CS+R and CS+N were significantly larger than to the CS-, demonstrating a persistence of the conditioned fear response. A significant Stimulus x Trial interaction was observed for the comparison between CS+R and CS- trials ($F(7, 161) = 5.70$, $p = .03$, $Eta^2 = .01$), but not between CS+N and CS- trials ($F(7, 161) < 1$, p = NS, $Eta^2 = .00$). Contrary to our hypothesis, there was no significant difference in the magnitude of the SCR to the CS+R versus CS+N. If anything, SCRs to the CS+R were slightly larger than those to the CS+N (see Figure 2, Panel B).

Reinstatement Phase (Day 3).

Responses to the CSs during the reinstatement and extinction phase, which immediately followed the unsignalled shock presentations, were initially examined across the ten presentations of each CS using a two-factor (Stimulus, Trials), repeated measures model. The Stimulus factor has three levels (CS+R, CS+N, CS-); the Trials factor has 10 levels. There was a non-significant trend for an effect of Stimulus ($F(2, 46) = 2.84$, $p = .07$, $Eta^2 = .01$), as well as a significant Stimulus x Trials interaction ($F(18, 414) = 1.72$, $p = .03$, $Eta^2 = .02$). In order to decompose the interaction effect, we performed two-factor, mixed model repeated measures ANOVA with the Stimulus factor having 2 levels for each of 3 ANOVAs that compared CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials; the Trials factor had 10 levels. As can be seen in Table 1, there was a near significant main effect for the comparison of SCR magnitude for the CS+R and CS- trials, but not for the comparisons between CS+N and CS- trials or between the CS+R and CS+N trials. Significant Stimulus x Trial interactions were observed for comparisons between the CS+N and CS- ($F(9, 207) = 1.99$, $p = .04$, $Eta^2 = .02$) and between the CS+R and CS+N ($F(9, 207) = 1.97$, $p = .05$, $Eta^2 = .02$), but not between the CS+R and CS- ($F(9, 207) = 1.18$, $p = .31$, $Eta^2 = .01$). As can be seen in Figure 2, the significant interaction for the comparison between CS+R and CS+ N trials reflected somewhat larger SCRs to initial CS+R presentations and then a reversal whereby there were somewhat larger SCRs to the later CS+N presentations. We also performed two-factor (Stimulus, Trials), mixed model repeated measures ANOVA that only considered the first two presentations of each CS during the reinstatement phase, so as to avoid the potential dampening of differences in reactivity to the CSs due to rapid extinction. The Stimulus factor has 2 levels in each of 3 analyses that compared CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials; the Trials factor has 2 levels. As can be seen in Table 1, the SCRs to CS+R and CS+N presentations were significantly larger than SCRs to CS- presentations; however, there was no significant difference between SCRs to the CS+R and CS+N presentations.

Re-extinction Phase (1 Month).

Responses to the CS+R, CS+N and CS- trials during the re-extinction phase were analyzed using two-factor (Stimulus, Trials) repeated measures model with the Stimulus factor having 3 levels (CS+R, CS+N, CS-) and the Trial factor having 8 levels. There was no significant Stimulus main effect ($F(2, 32) = 1.21$, $p = .31$, $Eta^2 = .01$) as well as no significant Stimulus x Trials interaction ($F(18, 288) = 1.45$, $p = .11$, $Eta^2 = .03$) (Figure 2, Panel C).

Re-acquisition Phase (1 Month).

Responses to the CSs during the re-acquisition phase were examined across the 8 presentations of each CS using a two-factor (Stimulus, Trials) repeated measures model with the Stimulus factor having 2 levels for each of 3 ANOVAs comparing CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials and the Trials factor with 8 levels. As can be seen in Table 1 and Figure 2, Panel C, we observed a pattern similar to that for acquisition on day 1. Reactivity to CS+R and CS+N was markedly stronger when compared to their respective CS- trials, but there was no significant difference in the magnitudes of SCRs to the CS+R and CS+N. There was a significant Stimulus x Trial interaction when comparing CS+R to CS- ($F(7, 111) = 5.00$, $p < .001$, $Eta^2 = .05$) and CS+R to CS+N ($F(7, 111) = 2.53$, $p = .02$, $Eta^2 = .03$), but not when comparing CS+N to CS- ($F(7, 112) = 1.32$, $p = .25$, $Eta^2 = .02$). As can be seen in Figure 2, Panel C, the

pattern of CRs to CS+R (blue) and CS+N (red) across trials is similar, although the CS+R elicited an exceptionally large response on the third presentation.

Discussion

Brief clips of moving tarantulas depicted within various contexts served as the conditioned stimuli for fear-conditioned SCRs. The differential conditioning procedure, combined with setting a differential conditioning threshold for SCRs, produced robust responses to the stimuli that would subsequently serve as the reactivated (CS+R) and non-reactivated (CS+N) cues, by which reconsolidation blockade of conditioned fear responses was assessed. Differential SCRs to the CS+R and CS+N, compared to their respective CS- presentations, remained largely intact and significant across subsequent testing of renewal and reinstatement on day 3) and reacquisition (4-weeks).

On the day following fear conditioning, reconsolidation blockade of the CR was attempted by administering propranolol followed by a single presentation of one of the two CS+s (i.e., the CS+R). On subsequent testing, we found that propranolol had no measurable reconsolidation-blocking effect on the fear-conditioned SCR, in that no significant differences between SCRs to the reactivated and non-reactivated CS+ were observed on either of the post-reactivation visits. In other words, administration of propranolol prior to reactivation of the CS+R failed to diminish the targeted fear memory trace.

Our negative findings are consistent with those reported by Soeter and Kindt (2010) for SCR, who employed fear-relevant stimuli within a differential fear-conditioning paradigm to assess the impact of pre-reactivation propranolol on SCR, as well as fear-potentiated startle. Soeter and Kindt observed a selective and complete abolishment of the fear-potentiated startle response, but no significant effect on SCR. Studies that have examined the effects of benzodiazepines on acoustic startle responses have also observed reductions in fear-potentiated startle in the absence of an effect on SCRs (e.g., Graham et al., 2005). The negative findings for SCR, yet previous positive findings for fear-potentiated startle, are noteworthy. Soeter and Kindt interpreted this discrepancy as indicative of distinct neural substrates that govern separate memory systems, i.e., declarative and procedural. Specifically, they suggested that SCR reflects declarative memory governed by the hippocampal complex, whereas fear-potentiated startle measures fear that is principally linked to amygdala activity. The finding that subjective reports of US expectancy (i.e., declarative knowledge) were also unaffected by propranolol was taken by Soeter and Kindt as further support that the fear-conditioned SCR reflected declarative memory.

Contrary to Soeter and Kindt's (2010) interpretation, there is substantial evidence that fear-conditioned SCRs represent something more than declarative knowledge. For example, multiple studies have clearly demonstrated fear-conditioned SCRs to masked stimuli (see Esteves, Parra, Dimberg, & Ohman, 1994; Flykt, Esteves, & Ohman, 2007), suggesting that SCRs can also be linked to non-declarative memory systems. Furthermore, conditioned SCRs have been linked to metabolic activity in the fear circuit. In a study of human fear conditioning, Agren and colleagues (Agren et al., 2012) manipulated extinction by altering the amount of time following the first extinction trial, so as to examine the effect of a short versus long delay on reconsolidation blockade of the fear memory. Participants underwent reactivation of the CR by a single presentation of the CS that lasted two minutes, followed by extinction trials that were delayed either ten minutes (active) or six hours (control). SCR magnitudess were significantly

reduced in the active, compared to control, group, and this reduction coincided with attenuation of blood oxygen level dependent (BOLD) activity in the basolateral amygdala. Furthermore, SCRs and BOLD activity of the amygdala were positively correlated in the days following delayed extinction for both groups.

In another study that examined SCR during neuroimaging, Williams et al. (2001) simultaneously recorded SC and fMRI while participants viewed stimuli consisting of neutral control and fearful faces. Consistent with the results of Agren et al. (2012), elicitation of an SCR by the fear stimuli coincided with heightened amygdala activity. Furthermore, Williams et al. only observed increased hippocampal activity when the fear stimuli *did not* elicit an SCR. Taken together, the above findings suggest that SCRs: a) are associated with activity of the amygdala complex, b) are not necessarily associated with hippocampal activity when triggered by fear-relevant stimuli, and c) can reflect processes in the absence of declarative knowledge.

Why then does fear-potentiated startle, but not SC, respond to reconsolidation blockade by propranolol in humans? Skin conductance is considered to be a relatively pure measure of sympathetic activity (Wallin, 1981), and those stimuli that activate the sympathetic nervous system, i.e., increase sympathetic arousal, will produce an SCR. In contrast, the acoustic startle reflex has been shown to be influenced by emotional valence, such that it is augmented and diminished in the presence of emotionally negative and positive stimuli, respectively (e.g., Hamm, Greenwald, Bradley, Cuthbert, & Lang, 1991). In an early study of startle potentiation in humans, Bradley, Cuthbert and Lang observed that, whereas the acoustic startle response was determined by stimulus valence, SCR reflected the amount of arousal generated by the stimulus (Bradley, Cuthbert, & Lang, 1990). More specifically, the startle response was augmented in the presence of an emotionally negative stimulus and diminished in the presence of a positive stimulus, whereas SCR was increased to both positive and negative stimuli. The different findings for fear-potentiated startle and SCR pertaining to reconsolidation blockade suggest that propranolol can interfere with reconsolidation of the negative emotional valence associated with a fear-conditioned memory but does not block reconsolidation of the sympathetic arousal generated by the stimulus. Thus, the process that maintains the arousal component of a conditioned fear response appears to be resistant to reconsolidation blockade by propranolol in healthy individuals. We believe that SCR, rather than eyeblink startle, may be a better index of the fear that is associated with PTSD.

Clinical studies that have examined the ability of propranolol to interfere with reconsolidation of a traumatic memory in individuals diagnosed with PTSD have provided some support for its efficacy (Brunet et al., 2008, 2014). Brunet and colleagues found that propranolol administered shortly after reactivation of an individual's traumatic memory reduced psychophysiologic responding, which included a measure of SC reactivity, during subsequent script-driven traumatic imagery in individuals with PTSD, compared to a placebo control group (Brunet et al., 2008). It is worth noting that this clinical work used a more prolonged re-activation of the fear cue, as well as a combination of short-acting and long-acting propranolol, compared to the present study and those of Soeter and Kindt (Soeter & Kindt, 2010, 2011), which used a relatively brief presentation of the fear CS and a single 40 mg dose of short-acting propranolol. It may be that a longer CS presentation and/or more enduring propranolol dosage are needed to interfere with reconsolidation of the fear-conditioned SCR. If propranolol is to be pursued as a candidate intervention in reconsolidation blockade, future conditioning studies might consider

increasing the dosage across all participants (i.e., >40mg) or adjusting the dosage based on participant weight, so as to amplify the antagonistic effects of the drug.

It is possible that in our efforts to generate stronger differential conditioning that would be more resistant to a floor effect (i.e., use of an SPQ-15 cutoff score, more salient stimuli, and a stringent conditioning criterion) we actually produced conditioning that was highly resistant to noradrenergic blockade. It may also be that the day 2 reactivation trial actually potentiated SCRs to CS+R and this potentiation masked the effects of noradrenergic blockade. Although this seems unlikely, a placebo reactivation condition would have helped determine whether such potentiation had occurred.

A primary goal of the present work was to develop a strong conditioning model that could be used to test the potential efficacy of new candidate reconsolidation-blockade interventions in readily available normal samples before conducting more expensive and challenging clinical trials. It seems desirable to develop a model that will produce the most resilient fear conditioning that is ethically justifiable, particularly if the results are to be translated to a disorder characterized by over-consolidated fear memories, as is PTSD (Pitman, 1989). Despite the negative results obtained, we believe that our novel paradigm fulfills these criteria and may represent a viable model within which to test and compare reconsolidation blocking interventions. However, future investigations must also consider the observed discrepancies in how different indices of fear conditioning, such as fear-potentiated startle and SCR, respond to reconsolidation blockade. Moreover, we should strive to reconcile contradicting results across comparable tests of reconsolidation blockade (e.g., Kindt & Soeter, 2013 and Schiller et al., 2010). Efforts must be directed to better understand the cause and clinical impact of such findings.

Table 1: Results of mixed-model repeated measures ANOVA of SCR (√μS) for the CS interval.

Day	Test Phase	Comparison	DF	F-value	P	Eta^2
Day 1	Acquisition	CS+R vs CS-	1, 23	87.4	<.001	**0.27**
		CS+N vs CS-		43.71	<.001	**0.20**
		CS+R vs CS+N		1.11	0.30	**0.01**
Day 3	Renewal	CS+R vs CS-	1, 23	21.09	0.001	**0.17**
		CS+N vs CS-		14.85	<.01	**0.13**
		CS+R vs CS+N		<1	NS	**0.00**
	Reinstatement (2 trials)	CS+R vs CS-	1, 23	7.78	0.01	**0.06**
		CS+N vs CS-		7.48	0.01	**0.08**
		CS+R vs CS+N		<1	NS	**0.00**
	Reinstatement and Extinction (10 trials)	CS+R vs CS-	1, 23	3.73	0.07	**0.01**
		CS+N vs CS-		2.9	0.10	**0.01**
		CS+R vs CS+N		<1	NS	**0.00**
1 month	Re-acquisition	CS+R vs CS-	1,16	19.74	<.001	**0.07**
		CS+N vs CS-		9.48	<.01	**0.07**
		CS+R vs CS+N		<1	NS	**0.00**

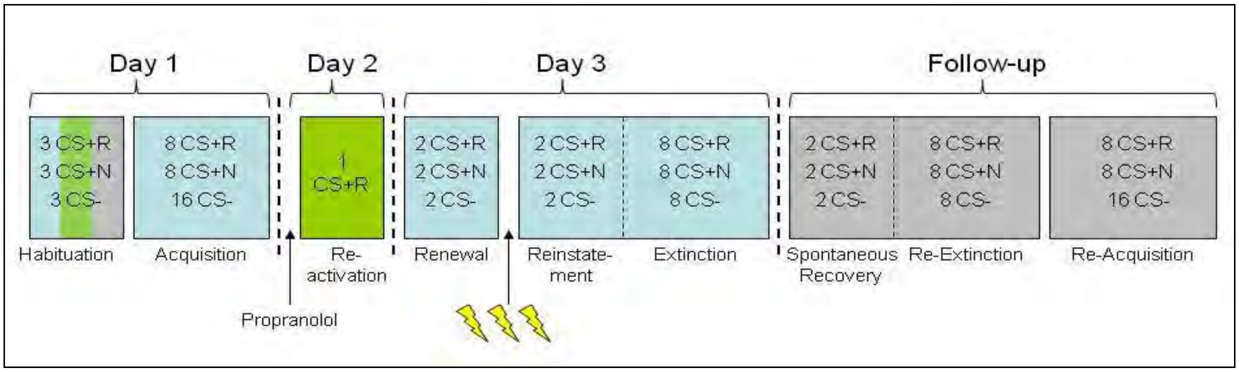

Figure 1: Depiction of the 4-session fear-conditioning procedure. Fear conditioning occurred on day 1. A single dose of 40 mg of propranolol was administered on day 2, followed by reactivation of one of the two the CS+s (i.e., the CS+R). On day 3 and the 1-month follow-up visit, post-intervention reactivity to the conditioned stimuli was tested. CS+R = CS+ to-be-reactivated; CS+N = CS+ non-reactivated; CS- = unreinforced. Lightning bolts represent unsignalled presentations of the US alone. Shading colors represent the context in which the stimuli were presented: blue = A, green = B, grey = C. CS+R and CS+N acquisition and re-acquisition trials occurred in blocks as described in the text.

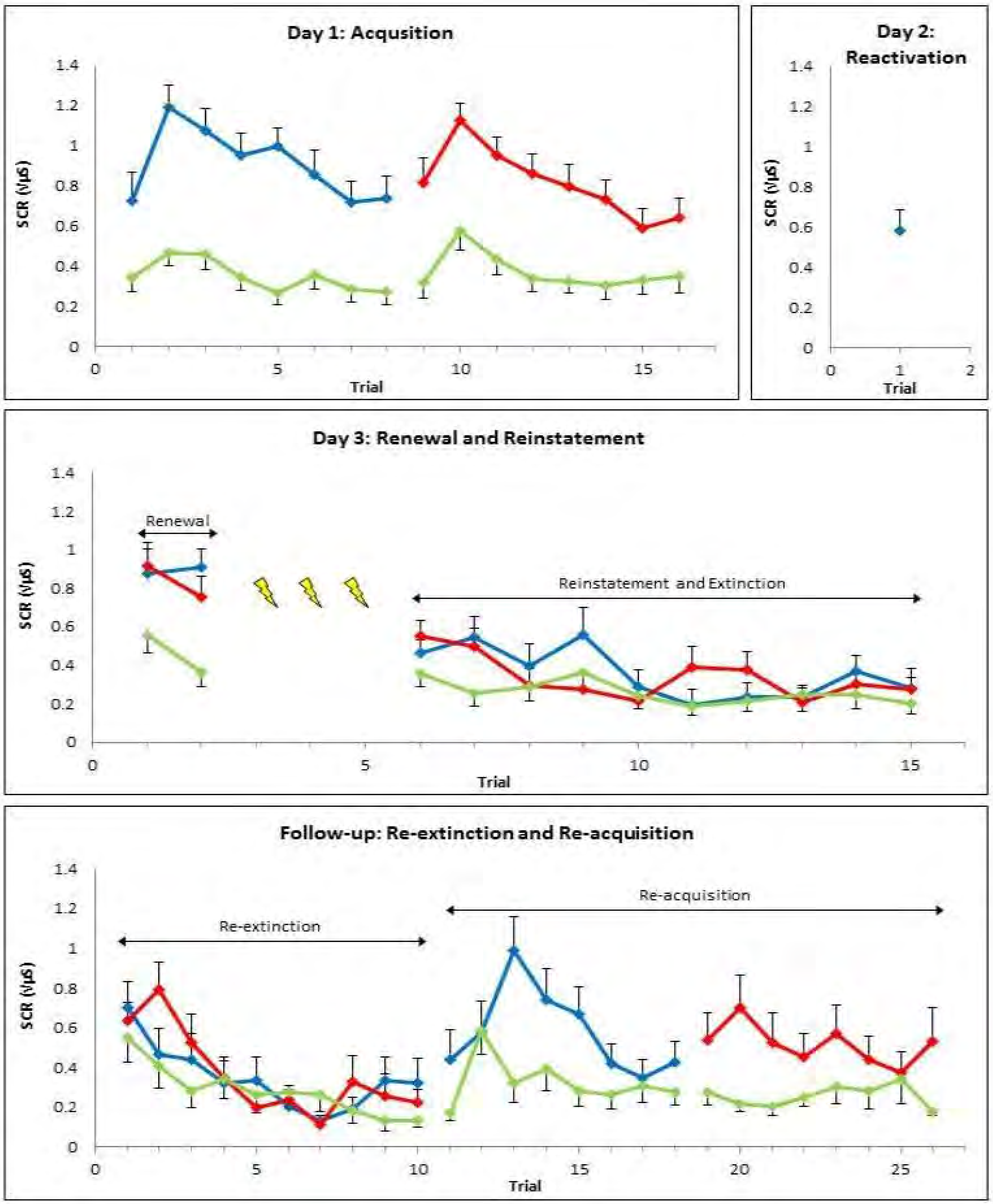

Figure 2. Group mean skin conductance responses to CS+R, CS+N, and CS- trials for the Acquisition (day 1), Reactivation (day 2), Renewal and Reinstatement (day 3), and Re-extinction and Re-acquisition (1 month) phases. Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Lightning bolts represent unsignalled presentations of the US alone. SCR = skin conductance response.

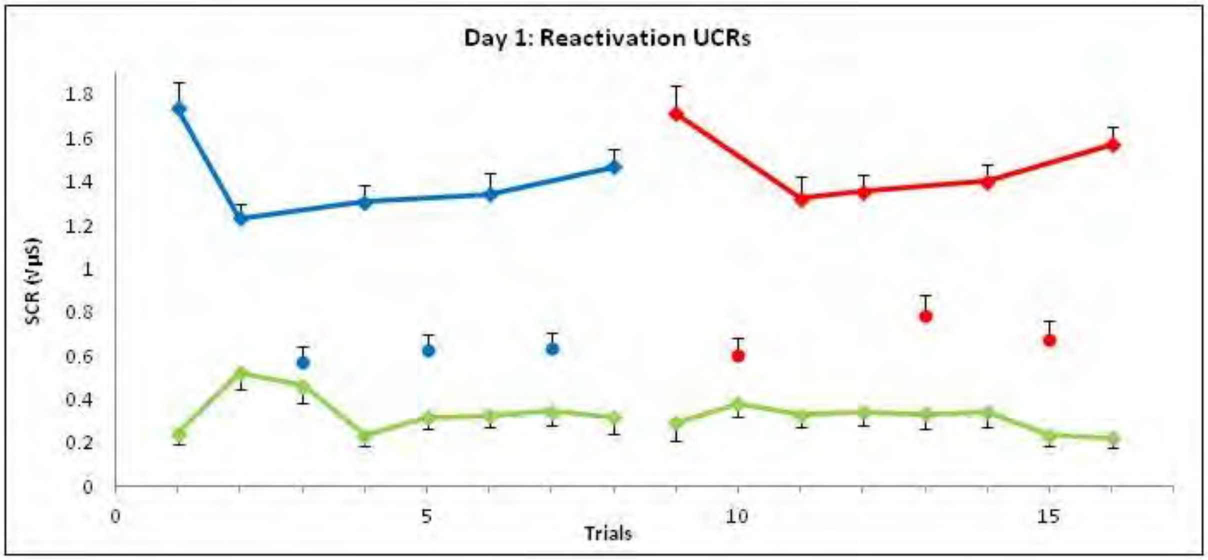

Figure 3. Group mean skin conductance responses during US interval following CS+R, CS+N, and CS- trials for the Acquisition phase (day 1). Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Unconnected circles represent unreinforced (i.e., no shock) CS+ trials. SCR = skin conductance response.

STUDY 2: BEHAVIORAL INTERVENTION

Delayed Extinction Fails to Reduce Skin Conductance Reactivity to Fear-Conditioned Stimuli

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Abstract

A brief ten minute time delay between an initial and subsequent exposures to extinction trials has been found to impair memory reconsolidation in fear-conditioned rodents and humans, providing a potential means to reduce fearfulness in anxiety disorders and posttraumatic stress disorder (PTSD). The present study used videos of biologically prepared, conditioned stimuli (tarantulas) to test the efficacy of delayed extinction in blocking reconsolidation of conditioned fear in healthy young adults. Strong differential conditioning, measured by skin conductance, was observed among a screened subset of participants during acquisition. However, the delayed-extinction intervention failed to reduce reactivity to the conditioned stimulus paired with the extinction delay. These results are consistent with other recent, mixed findings and point to a need for testing other candidate interventions designed to interfere with the reconsolidation process.

Delayed Extinction Fails to Reduce Skin Conductance Reactivity to Fear-Conditioned Stimuli

Fear memories like those associated with posttraumatic stress disorder (PTSD) must be consolidated (i.e. stabilized) in order to persist. PTSD is characterized by the presence of debilitating symptoms associated with persistent memories linked to the traumatic event. Two distinct memory consolidation mechanisms have been proposed: a short term synaptically-mediated process and a longer term hipocampally-mediated one (Dudai, 2004). While the former process is potentially vulnerable to intervention within minutes to hours following the traumatic event, it has proven difficult to intervene clincially before intial consolidation occurs; studies attempting this have reported mixed results (Hoge et al., 2012; Holmes, James, Coode-Bate, & Deeprose, 2009; Krauseneck et al., 2010; Nugent et al., 2010; Pitman et al., 2002; Stein, Kerridge, Dimsdale, & Hoyt, 2007; and Vaiva et al., 2003). Research using animals has shown that reactivation of a consolidated memory returns it to a destabilized state, from which it must be restabilized (i.e. reconsolidated) if it is to persist (Debiec & Ledoux, 2004; Nader, Schafe, & Le Doux, 2000; Nader & Einarsson, 2010). Reactivation of a trauma memory and subsequent interference with its *reconsolidation* may offer a feasible and effective treatment strategy. Reconsolidation is governed by neurobiological processes similar to those of consolidation (Lee, Everitt, & Thomas, 2004), and is susceptible to pharmacological blockade (Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Pitman et al., 2011; Przybyslawski, Roullet, & Sara, 1999; Kindt, Soeter & Vervliet, 2009). Studies exploring the efficacy of behavioral intervention, such as modifications to extinction learning within the reconsolidation window, have produced mixed results regarding interference with fear memory reconsolidation.

Brief re-exposure to a previously conditioned stimulus *prior* to the start of traditional extinction trials has been shown to interfere with the retention of the acquired fear response. For example, Monnfils et al. (2009) conditioned rats to a fear cue and 24 hours later provided all with a single exposure to the fear CS alone. Extinction training was then carried out either within or beyond a 6-hour window, the period of time during which reconsolidation is thought to be viable (Duvarci et al., 2004). The fear response was subsequently elicited only in animals that underwent extinction beyond that 6-hour reconsolidation window. That is, subsequent to a single exposure to the unpaired CS, rats that underwent extinction within the reconsolidation window did not show subsequent recovery of the fear response.

Schiller et al. (2010) extended this delayed-extinction approach to humans. Three groups of subjects underwent conditioning of skin conductance response (SCR) to a neutral CS+ (consisting of a colored square) that was paired with a mild shock to the wrist on 38% of trials. A differently colored square (CS-) was never paired with the shock. One day later, all subjects underwent extinction training in which both CSs were repeatedly presented without the unconditioned stimulus. In one group the fear memory was reactivated by a single presentation of the CS+, 10 minutes prior to the start of extinction. A second group was similarly reminded 6 hours before extinction. A third, control, group had no exposure to the CS+ reminder. Twenty-four hours following extinction, spontaneous recovery was assessed from SCRs to the CS+ alone) and was evident in the latter two groups, but not in subjects who had the CS+ reminder 10 minutes prior to the start of extinction. A subset of subjects were re-examined one year later with a re-instatement phase consisting of exposure to 4 unanticipated shocks followed by presentations of the CS+ alone. There was successful reinstatement of the conditioned SCR to the CS+ in the 6-

hour and no-exposure control groups, but not in the subjects that were previously reminded of the CS+ 10 minutes prior to extinction. Most remarkably, the disruptive effect associated with a single CS+ alone exposure had persisted for one year.

Agren et al. (2012) conditioned a fear SCR in humans on Day 1 and then re-activated the fear memory on Day 2 by presenting the CS+ for two minutes. Subjects underwent extinction either 10 minutes or 6 hours later. On Day 3 subjects underwent a renewal session with no new shocks delivered during fMRI scanning. Increased fear responding, as measured by SCR, was observed in the 6-hour, but not the 10-minute, group. They further demonstrated that the conditioned fear memory had been localized to the basolateral amygdala as seen by signal activation on fMRI, and that patterns of neural activity could predict the return of fear in subjects who underwent undisrupted reconsolidation.

The role of CS quality on reconsolidation extinction effects was examined by Golkar, Bellander, Olsson and Ohman (2012). In this study, human subjects underwent conditioning with fear-relevant and fear-irrelevant CS+'s, followed by extinction and reinstatement testing on consecutive days. Exposure to a single presentation of one of the CS+s alone, 10 minutes prior to extinction, failed to prevent the return of extinguished fear, as measured by SCR. This was the case for the fear-irrelevant, as well as the fear-relevant, CS+. Reconsolidation disruption by delayed extinction was partially supported by Soeter and Kindt (2011), who conditioned 40 undergraduate subjects on Day 1 to two fear-relevant CS+s with a fear-irrelevant stimulus serving as the CS-. SCR, fear-potentiated startle and subjective expectancy ratings served as the dependent measures. Extinction took place on Day 2, 10 minutes after exposure to one of the CS+s. On Day 3, subjects underwent a re-extinction phase followed by a reminder shock that was intended to maximize the likelihood of fear memory expression, followed by a re-acquisition phase. Spontaneous recovery was found to be selectively prevented by prior CS+ exposure. However, after exposure to a series of unsignaled shocks, reinstatement, and savings on re-acquisiton were readily demonstrated, indicating that prior exposure to the CS+ had not eradicated the fear memory. The authors questioned whether the positive findings reported by Shiller et al., (2010), were due to the elimination of the first trial in their test of fear retention at one year. Kindt and Soeter (2013) re-studied the impact of a single fear-relevant CS+ expsoure on fear retention of SCR, startle repsonse and self-report of US-expectancy in a new sample of 40 subjects. They found that exposure to the unpaired CS+ during a 10 minute window prior to exinction training did not attentuate later recovery of either extinguished startle fear responding, SCR or US-expectanacy ratings.

The present study examined the effect of reactivating a fear memory, prior to initiating a series of extinction trials, on reduction of fear-memory reconsolidation. A differential conditioning procedure was used to establish conditioned SCRs to two different fear-relevant CS+s; a day later one of the two CS+s was reactivated by a single presentation 10 minutes prior to initiating a series of extinction trials of both CS+s. Reconsolidation blockade was tested on the following day (day 3) by examining spontaneous recovery and reinstatement of the conditioned SCR. In an attempt to establish fear-conditioned SCRs that are more resistant to extinction and that might serve to test the relative strengths of various drug and non-drug candidates for reconsolidation blockade, we employed more highly prepared CSs, more fear-sensitive subjects, and stronger conditioned responses (CRs), as done previously by Spring et al. (2015). It has been shown that certain classes of CSs, when paired with a US, produce a stronger fear CR, i.e., they

are more “prepared” to enter into an association with the US (Mineka & Öhman, 2002). We enhanced preparedness of the CSs by using 12-sec, high-definition video clips of three crawling tarantulas, each conspicuously different in appearance. Second, we limited enrollment to participants who scored approximately one standard deviation or greater above the population mean on the Spider Phobia Questionnaire-15 (SPQ-15), as described by Olatunji, Woods, de Jong, Teachman, Sawchuk and David (2009). However, we did exclude anyone who endorsed symptoms of clinical spider phobia (see Method). Third, we required that participants show evidence of strong differential conditioning, as determined by a more stringent cutoff assigned to the CRs recorded during Day 1 acquisition (specified below). Participants with subthreshold CRs were withdrawn after Day 1.

Method

Participants

Prior to enrollment, participants were screened by phone to verify the presence of a manageable, non-phobic fear of spiders as determined by scores above the mean on the SPQ-15 (Olatunji et al., 2009) and phobia criteria extracted from the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-IV; First, Spitzer, Gibbon, & Williams, 1997). Participants underwent a set of screening criteria taken directly from the SCID-IV to verify absence of current psychiatric disorders, serious medical or neurological conditions, brain injury, and current or past substance abuse. A positive response to a screening criterion led to a full examination of that criterion per SCID-IV. A urine drug screen verified the absence of illicit substances and psychotropic medications. The presence of a current Axis I psychiatric disorder or illicit substances/medications was grounds for withdrawal from the study.

Sixty-six healthy participants (41 females, 25 males) were enrolled in the study. Of these, none had an unmeasurable (very low) SC level or a current Axis I psychiatric disorder. Following the Day 1 procedure, 45 were withdrawn due to: data collection problems ($n = 9$), drop out ($n = 1$), and failure to demonstrate adequate differential conditioning ($n = 35$, conditioning criteria are described below in Data Reduction). The remaining 21 (10F, 11M) who underwent study procedures on Days 1-3 had a mean age of 23.05 years ($SD = 2.58$, range 18 to 28 years) and a mean score on the SPQ-15 of 7.55 ($SD = 2.02$, range 4 to 12 of a possible 0 to 15). Mean years of education was 15.20 ($SD = 1.64$, range 13 to 17 years). Five of the 21 participants were not included in the Day 30 analyses due to: data collection error ($n = 1$), and being lost to follow-up ($n = 4$).

The study protocol was approved by the Partners Human Research Committee (PHRC), as well as the United States Army Medical Research and Materiel Command (USAMRMC) Human Research Protection Office (HRPO). After a full explanation of the procedures, all participants provided written informed consent.

Equipment and Stimuli

Equipment and stimuli were the same as those used by Spring, Wood, Mueller-Pfeiffer, Milad, Pitman and Orr (2014). Skin conductance analog signals were recorded using a Coulbourn Lab Linc V Series Human Measurement System (Coulbourn Instruments, Whitehall, PA) with a Coulbourn Isolated Skin Conductance Coupler (V71-23) through 8mm (sensor diameter) Ag/AgCl electrodes (In Vivo Metric; Healdsburg, CA) filled with an isotonic paste. Electrodes were separated by 14mm, as determined by the width of the adhesive collar, and placed on the

hypothenar surface of the subject's non-dominant hand in accordance with published guidelines (Boucsein et al., 2012; Fowles et al., 1981). The SC signal was sampled at 1000 Hz and digitized by a Coulbourn Analog to Digital Converter (V19-16). A Cobalt notebook computer (IBM-compatible; Cobalt Computers, Whitehall, PA) with custom-designed software was used to record and store the digitized physiological signals.

Nine high-definition video clips (Virtually Better Inc., Decatur, GA) depicting one of three tarantulas occupying one of three contexts comprised the conditioned stimuli (CS). Two of the three tarantulas always served as either the to-be-reactivated CS+ (CS+R), which would have the 10-min delay following the first extinction trial on day 2, or the to-be-non-reactivated CS+ (CS+N), which would not have a 10-min delay following the first extinction trial on day 2. The CS+R and CS+N were paired with the unconditioned stimulus (US, shock) on day 1. The third tarantula served as the CS- and was not paired with the US. The three contexts within which the tarantulas appeared were a kitchen (A), bedroom (B) and office (C). The particular tarantula that served as the CS+N or CS+R was counterbalanced across participants; the tarantula used to represent the CS- was the same across subjects.

The US was a 0.5-sec mild electric shock ranged in intensity (0.2 to 4.0 milliamperes) according to the level determined by the participant to be "highly annoying but not painful." The US was delivered using a Coulbourn Transcutaneous Aversive Finger Stimulator (E13-22) through shock electrodes attached to the middle segments of the 2nd and 3rd fingers on the hand opposite to that on which the SC recording electrodes were attached.

Video clips lasted 12 seconds: four seconds of context alone (i.e., no tarantula), followed by eight seconds of context plus tarantula. On reinforced trials, the US immediately followed the CS+. The intertrial interval consisted of a black screen and was randomized to last 16, 18, 20, 22, or 24 seconds. The procedure was implemented using E-Prime Professional 2.0 (Psychology Software Tools, Inc., Sharpsburg, PA).

Procedure

As depicted in Figure 4, the procedure consisted of a differential fear-conditioning paradigm that entailed laboratory visits over three consecutive days and a one-month follow-up visit. On day 1, participants were instructed: *"Today, you will be viewing videos of spiders on the television, and you will receive electric shocks on your fingers after viewing some of the spiders. These shocks will be annoying, but not painful. We will also use electrodes on your palm to record how your body responds to this procedure."* Following these instructions, participants set the shock to a level to be “highly annoying but not painful” (Orr et al., 2000). Participants were then shown still images, i.e., screenshots, of the three tarantulas that would serve as CSs, accompanied by these instructions: *"During the experiment, it will be important that you are able to tell these spiders apart. To do this, try focusing on the legs. For this spider, note the alternating black and white stripe pattern. For this spider, note the orange highlights. For this spider, note that the legs are solid black."* Prior to beginning the procedure, the lights were dimmed and over-ear headphones placed on the participant to reduce ambient noise and enable communication with study staff in the next room. Participants were instructed to sit still in the chair, keep their eyes open, and be attentive to the stimuli presented on the screen. Next, there was a 5-min baseline period to record physiological levels.

Day 1 consisted of two sequential phases: 1) unreinforced presentations of each of the nine possible spider-context combinations in pseudorandom order (*habituation*), and 2) eight partially reinforced (i.e., five of eight) presentations each of CS+R and CS+N, presented separately in blocks and interspersed pseudorandomly with eight presentations of CS- (*acquisition*). Presentation order of CS+R and CS+N trial blocks was counterbalanced across participants. All CS+R, CS+N, and CS- presentations during acquisition occurred within context A. Participants who did not meet the defined cutoff for demonstrating a differential conditioned response (see below) were withdrawn prior to day 2.

The procedures for days 2, 3, and the 1-month follow-up were largely the same as for day 1, with the following exceptions: a) the procedure for setting the level of shock was not repeated, as the shock level determined on the first visit was used for the remainder of the study, b) participants were only familiarized with images of the stimuli prior to undergoing the day 1 procedure, and c) rather than "will receive" as on day 1, participants were instructed that they "may or may not receive" electric shocks.

Day 2 consisted of ten unreinforced presentations, i.e., extinction trials, each of the CS+R, CS+N and CS-. All stimuli were presented in context B. Reactivation of the CS+R was accomplished by initially presenting the CS+R and then waiting 10 min prior to presenting the remaining CS+R, CS+N and CS- extinction trials, which were interspersed.

Day 3 consisted of three sequential phases: 1) two unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*renewal test*); and 2) 3 unsignalled presentations of the US alone, followed by 3) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*reinstatement test trials* and *extinction*). All presentations of the CSs occurred in context A. Ordering of CS+R and CS+N presentations, within the full set of trials that included CS- presentations, was counterbalanced across subjects.

The one-month follow-up consisted of two phases: 1) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (2 *spontaneous recovery test* trials and 8 *re-extinction* trials), followed by 2) eight partially-reinforced, i.e., five of eight, presentations each of CS+R and CS+N presented in successive blocks and interspersed with eight CS- trials for the respective blocks as was done on day 1 (*re-acquisition/savings test*). All stimuli were presented in context C during this visit, and the CS+R block of trials was presented first.

Physiological Measures and Data Reduction

As previously described (Milad, Orr, Pitman, & Rauch, 2005; Orr et al., 2000), an SCR for the CS interval was calculated for each trial by subtracting the mean SC level during the two sec prior to CS onset (context alone presentation) from the peak SC level during the eight sec CS interval. These SCR values reflect change in skin conductance level beyond that resulting from presentation of context alone. A square root transformation was applied to the absolute value of each SCR, followed by replacement of the + or - sign, prior to statistical analysis.

For day 1, the untransformed SCR data were scored to determine whether a definable differential SCR was obtained for *both* the CS+R and CS+N during the acquisition phase. We averaged SCRs across respective CS+R, CS+N, and CS- trials in order to calculate a difference score between the CS+R and its respective CS- trials and between the CS+N and its respective

CS- trials. A cutoff of .1μS was applied to each difference score and participants with one or both difference scores below this cutoff were withdrawn from the study prior to Day 2.

Results

The primary statistical model was mixed-model, repeated measures analysis of variance (ANOVA), performed separately for each experimental phase. Participants were treated as a random effect, Stimulus (CS+R, CS+N, CS-) as a within-participants effect, and Trials as the repeated measure.

Acquisition Phase (day 1).

Responses to the CSs during the acquisition phase were examined across the eight presentations of each CS using a mixed-model repeated measures ANOVA with two factors: Stimulus (CS+R, CS- or CS+N, CS-) and Trials. As can be seen in Table 2 and Figure 5, Panel 1, during the acquisition phase, the CS+R and CS+N demonstrated comparably larger SCRs, compared to their respective CS- trials. The magnitude of the SCRs did not differ between CS+R and CS+N trials. There were significant Stimulus x Trials interactions for the comparisons of CS+R to CS- ($F(7,140) = 3.61$, $p = .001$, $Eta^2 = .03$), CS+N to CS- ($F(7,140) = 4.43$, $p < .001$, $Eta^2 = .03$) and CS+R to CS+N ($F(7,140) = 3.47$, $p = .002$, $Eta^2 = .03$).

SC responses for the US interval, which represented the unconditioned response (UR), were calculated and plotted for the acquisition phase (see Figure 6). Because SCR onset has a known latency of 1-2 s (Edelberg, 1967), the 1-s interval immediately following US onset was used as the baseline for calculating the SC UR, which was subtracted from the peak SC level within the 6-s interval following US onset to yield the UR. A square root transformation was applied to the UR, as was done for the CR. As expected, both CS+R and CS+N trials produced larger SCRs during the US interval, compared to CS- trials ($F(1,20) = 388.5$, $p < .001$, $Eta^2 = .49$; $F(1,20) = 360.0$, $p < .001$, $Eta^2 = .47$; respectively). There were significant Stimulus x Trials interactions for both CS+R and CS+N trials ($F(7,140) = 29.77$, $p < .001$, $Eta^2 = .13$; $F(7,140) = 39.78$, $p < .001$, $Eta^2 = .15$; respectively). The average UR magnitudes for CS+R and CS+N reinforced trials did not differ ($F(1,20) < 1$, p = NS). There was a significant Stimulus x Trials interaction for the comparisons of CS+R to CS+N trials ($F(7,140) = 26.10$, $p < .001$, $Eta^2 = .11$, Figure 6).

Delayed Extinction Phase (Day 2).

SCRs to the CSs during the delayed extinction phase were first examined across the 10 presentations of each CS using a two-factor (Stimulus, Trials), repeated measures model (see Figure 5, Panel 2). The Stimulus factor has three levels (CS+R, CS+N, CS-); the Trials factor has ten levels. The main effect of Stimulus ($F(2, 40) = 10.87$, $p < .001$, $Eta^2 = .05$) and the Stimulus x Trials interaction ($F(18, 360) = 2.29$, $p = .002$, $Eta^2 = .03$) were significant. The Stimulus main effect was explored by comparing CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials using two-factor (Stimulus, Trials), mixed model repeated measures ANOVA. For these analyses, the Stimulus factor has two levels in the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials and the Trials factor has ten levels. These analyses yielded significant Stimulus main effects for SCRs to the CS+R vs CS- ($F(1,20) = 18.09$, $p < .001$, $Eta^2 = .08$ and CS+N vs CS- ($F(1,20) = 10.63$, $p = .004$, $Eta^2 = .03$, but not for the comparison of CS+R vs CS+N ($F(1,20) = 2.91$, $p = .10$, $Eta^2 = .01$). The analyses also produced significant Stimulus x Trials interaction effects for CS+R vs CS- ($F(9,180) = 2.85$, $p = .004$, $Eta^2 = .03$) and CS+R vs CS+N ($F(9,180) =$

2.68, p = .006, Eta^2 = .02), but not for the comparison of CS+N vs CS- ($F(1,20)$ = 1.72, p = .09, Eta^2 = .03).

Renewal Phase (Day 3).

Responses to the CSs during the renewal phase were examined over the two presentations of each CS using a two-factor (Stimulus, Trials) repeated measures ANOVA. The Stimulus factor has two levels in each of three ANOVAs that compared CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials; the Trials factor has 2 levels. As can be seen in Table 2 and Figure 5, Panel 3, SCRs to the CS+R and CS+N were significantly larger than to the CS-, demonstrating a persistence of the conditioned fear response. The Stimulus x Trials interaction effects were not significant (F's(1, 20) < 1, p's = NS, Eta^2 = .00). Our prediction that the magnitude of the SCR would be smaller to the CS+R, compared to the CS+N, was not supported, as there was no difference; if anything, SCRs to the CS+R were slightly larger than those to the CS+N (see Figure 5, Panel 3).

Reinstatement Phase (Day 3).

Responses to the CSs during the reinstatement and extinction phase, which immediately followed the unsignalled shock presentations, were first examined across the ten presentations of each CS using a two-factor (Stimulus, Trials), repeated measures model. The Stimulus factor has three levels (CS+R, CS+N, CS-); the Trials factor has ten levels. The main effect of Stimulus ($F(2, 40)$ = 2.14, p = .13, Eta^2 = .01) and the Stimulus x Trials interaction ($F(18, 360)$ = 1.51, p = .08, Eta^2 = .02) were not significant. For the interested reader, direct comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials are provided in Table 2. As can be seen in the table, the comparison of CS+R to CS- SCR magnitude yielded a nearly significant difference, whereas the comparison of CS+N to CS-trials did not. However, the comparison of CS+R and CS+N trials indicated that SCR magnitudes did not differ. In order to avoid the potential dampening of differences in SCR to the CSs due to rapid extinction, we also performed two-factor (Stimulus, Trials), mixed model repeated measures ANOVA that only considered the first two presentations of each CS during the reinstatement phase. For these analyses, the Stimulus factor has two levels in the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials and the Trials factor has two levels. As can be seen in Table 2, the SCRs to CS+R tended to be slightly larger than those to the CS-, whereas there was no difference when comparing CS+N and CS- trials. However, there was no difference between SCR magnitude to the CS+R and CS+N presentations.

Re-extinction Phase (1 Month).

Spontaneous recovery of the conditioned SCR was assessed by examining response magnitudes of the first two CS+R, CS+N and CS- trials during the re-extinction phase using two-factor (Stimulus, Trials) repeated measures model; the Stimulus factor has three levels (CS+R, CS+N, CS-) and the Trials factor has two levels. This analysis produced a nearly significant Stimulus main effect ($F(2, 30)$ = 3.14, p = .06, Eta^2 = .03). The Stimulus x Trials interaction was not significant ($F(2, 30)$ < 1, p = NS, Eta^2 = .00). In order to further explore the Stimulus main effect, we compared SCR magnitudes of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials using two-factor (Stimulus, Trials), mixed model repeated measures ANOVA. For these analyses, the Stimulus factor has two levels in the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials and the Trials factor has two levels. As can be seen in Table 1, consistent with predictions, SCRs to CS+N were significantly larger than those to the CS-, whereas there was no difference when comparing the initial CS+R to CS- trials. The

interpretation of this pattern is complicated by the absence of a difference between SCR magnitudes for CS+R and CS+N trials when directly compared. When SCRs were examined across all re-extinction trials, there was no significant Stimulus main effect ($F(2, 30) < 1$, p = NS, $Eta^2 = .00$), as well as no significant Stimulus x Trials interaction ($F(18, 270) = 1.19$, $p = .27$, $Eta^2 = .03$) (Figure 5, Panel 4).

Re-acquisition Phase (1 Month).

Responses to the CSs during the re-acquisition phase were examined across the eight presentations of each CS using a two-factor (Stimulus, Trials) repeated measures model with the Stimulus factor having two levels for each of 3 ANOVAs comparing CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials and the Trials factor with eight levels. As can be seen in Table 1 and Figure 5, Panel 4, we observed a pattern somewhat similar to that for acquisition on day 1. Reactivity to CS+R and CS+N was stronger when compared to their respective CS- trials, but there was no significant difference in the magnitudes of SCRs to the CS+R and CS+N. The Stimulus x Trials interaction was not significant when comparing CS+R to CS- ($F(7, 105) = 1.77$, $p = .10$, $Eta^2 = .03$) and CS+R to CS+N ($F(7, 105) = 1.28$, $p = .27$, $Eta^2 = .02$), and marginally significant when comparing CS+R to CS+N ($F(7, 112) = 1.97$, $p = .07$, $Eta^2 = .03$).

Discussion

Brief clips of moving tarantulas depicted within various contexts served as the conditioned stimuli for fear-conditioned SCRs. As observed by Spring et al. (2015), the differential conditioning procedure, combined with setting a differential conditioning threshold for SCRs, produced robust responses to the stimuli that would subsequently serve as the reactivated (CS+R) and non-reactivated (CS+N) cues, by which reconsolidation blockade of conditioned fear responses was assessed. Differential SCRs to the CS+R and CS+N, compared to their respective CS- presentations, remained largely intact and significant across subsequent testing of renewal and reinstatement on day 3) and reacquisition (4-weeks).

On the day following fear conditioning, reconsolidation blockade of the fear conditioned SCR was attempted by providing a single exposure to the CS+R alone. On subsequent testing, we found that an extinction delay of 10 min had no measurable reconsolidation blocking effect on the fear-conditioned SCR. Specifically, no significant differences between SCRs to the CS+R (with 10-min. extinction delay) and the CS+N (without a delay) were observed on either of the post-reactivation visits. An initial delay of 10 minutes between the first extinction trial and subsequent trials failed to diminish the targeted fear memory trace.

The failure to "update" and interfere with the reconsolidation process using a non-pharmacological intervention observed in the present study is at odds with the findings of Shiller et al., (2010), and Agren et al., (2012), but consistent with prior negative results from Soeter and Kindt (2011), Kindt and Soeter (2013), Golkar et al. (2012) and Oyarzun et al. (2012). The explanation for the inconsistent findings across studies is not readily apparent. The lack of consistency in findings seems unlikely to be due to procedural differences across studies (see Golkar et al., 2012). It may be that a single extinction delay, as used in the present study and by other investigators, is a weak intervention for strongly conditioned fear responses and thereby produces unreliable results. Failure to reduce fear to the reactivated and non-reactivated CS+s in the present study may be due, at least in part, to the combined use of prepared, fear-relevant stimuli and selection of subjects who demonstrated strong SCR conditionability and reported a

predisposition to being afraid of spiders. As noted in Spring et al. (2015), it is possible that in our efforts to produce differential conditioning that would be more resistant to a floor effect we actually produced conditioning that was highly resistant to intervention. The strength of the conditioned SCR to the reactivated CS+ may have been so strong that a single exposure to the CS+R, unpaired with the US, was simply inadequate to produce reconsolidation blockade. Studies using animals have demonstrated that stronger memories are more resistant to the reconsolidation process (Suzuki, Josselyn, Frankland, Massushinge, Silva & Kida., 2004; Wang, Alvares & Nader, 2009). Future studies that systematically explore the frequency and/or duration of the CS+ alone exposure prior to the extinction phase could help determine if a there is a threshold that has to be surmounted for the reconsolidation window to effectively “open”.

An additional explanation for the present study’s failure to produce reconsolidation blockade is suggested by Golkar et al.’s (2012) discussion of their negative reconsolidation findings. They point out that memory updating, which is required for reconsolidation, is most likely to occur when the reactivation trial presents novel information. This has been found to be the case for both animals and humans (see Lee, 2009 for review). The present study, as has much of the reconsolidation work to date, used a partial reinforcement schedule for pairing of the CS+s and unconditioned stimulus. Consequently, a single unreinforced reactivation trial does not provide any new information to the subject, as it may simply have been another of several unreinforced CS+ trials. As Golkar et al. point out, if a 100 percent reinforcement schedule had been used, then the unreinforced reactivation trial would have provided new information to the subject, viz., that the CS+ may no longer predict the US, and thereby would cause the memory to become destabilized and amenable to blockade.

A primary goal of our work has been to develop a conditioning model that could be used to test the potential efficacy of new candidate reconsolidation-blockade interventions in normal samples before moving on to expensive and challenging clinical trials. Despite the negative results obtained here and by Spring et al., we believe that our conditioning protocol fulfills these criteria and may represent a viable model within which to test and compare reconsolidation blocking interventions.

Table 2: Results of mixed-model repeated measures ANOVA of SCR ($\sqrt{\mu S}$) for the CS interval.

			DF	F	p	Eta^2
Day 1	Acquisition	CS+R vs CS-	1, 20	70.24	<.001	0.19
		CS+N vs CS-		75.13	<.001	0.28
		CS+R vs CS+N		<1	NS	0.00
Day 3	Renewal	CS+R vs CS-	1, 20	25.8	<.001	0.26
		CS+N vs CS-		25.63	<.001	0.20
		CS+R vs CS+N		2.55	0.13	0.03
	Reinstatement (2 trials)	CS+R vs CS-	1, 20	3.64	0.07	0.05
		CS+N vs CS-		<1	NS	0.01
		CS+R vs CS+N		<1	NS	0.02
	Reinstatement and Extinction (10 trials)	CS+R vs CS-	1, 20	4.30	0.05	0.01
		CS+N vs CS-		1.45	0.24	0.00
		CS+R vs CS+N		<1	NS	0.00
1 Month	Re-extinction Spontaneous recovery (2 trials)	CS+R vsCS-	1,15	2.33	0.15	0.03
		CS+N vs CS-		6.08	0.03	0.07
		CS+R vs CS+N		<1	NS	0.02
	Re-acquisition	CS+R vs CS-	1,15	12.39	0.003	0.05
		CS+N vs CS-		3.48	0.08	0.02
		CS+R vs CS+N		1.29	0.27	0.01

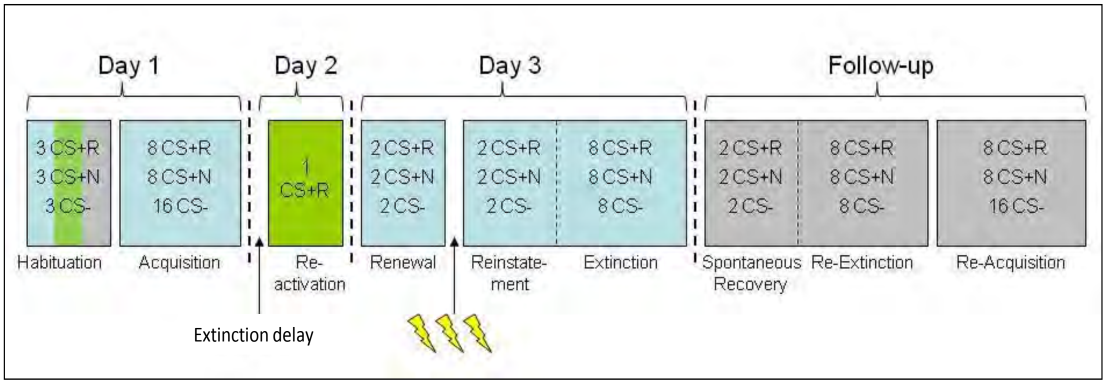

Figure 4: Depiction of the 4-session fear-conditioning procedure. Fear conditioning occurred on day 1. A single reactivation trial of one of the two CS+s (i.e., the CS+R) was followed by a 10-min delay and then a series of extinction trials for the CS+R, CS+N and CS-. On day 3 and the 1-month follow-up visit, post-intervention reactivity to the conditioned stimuli was tested. CS+R = CS+ to-be-reactivated; CS+N = CS+ non-reactivated; CS- = unreinforced. Lightning bolts represent unsignalled presentations of the US alone. Shading colors represent the context in which the stimuli were presented: blue = A, green = B, grey = C. CS+R and CS+N acquisition and re-acquisition trials occurred in blocks as described in the text.

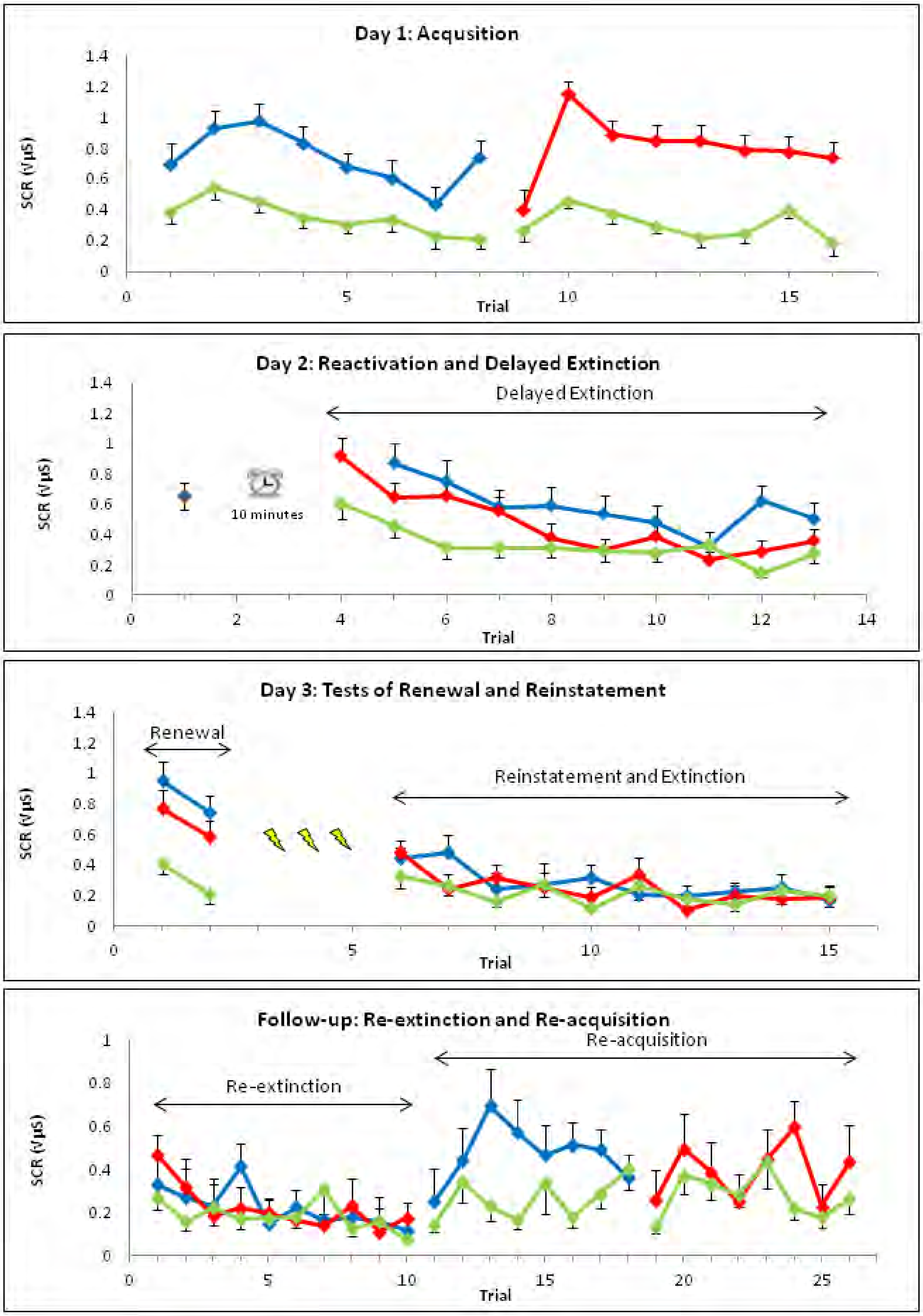

Figure 5. Group mean skin conductance responses to CS+R, CS+N, and CS- trials for the Acquisition (day 1), Reactivation and Delayed Extinction (day 2), Renewal and Reinstatement (day 3), and Re-extinction and Re-acquisition (day 30) phases. Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Lightning bolts represent unsignaled presentations of the US alone. Alarm clock represents 10 minute delay without stimulus. SCR = skin conductance response.

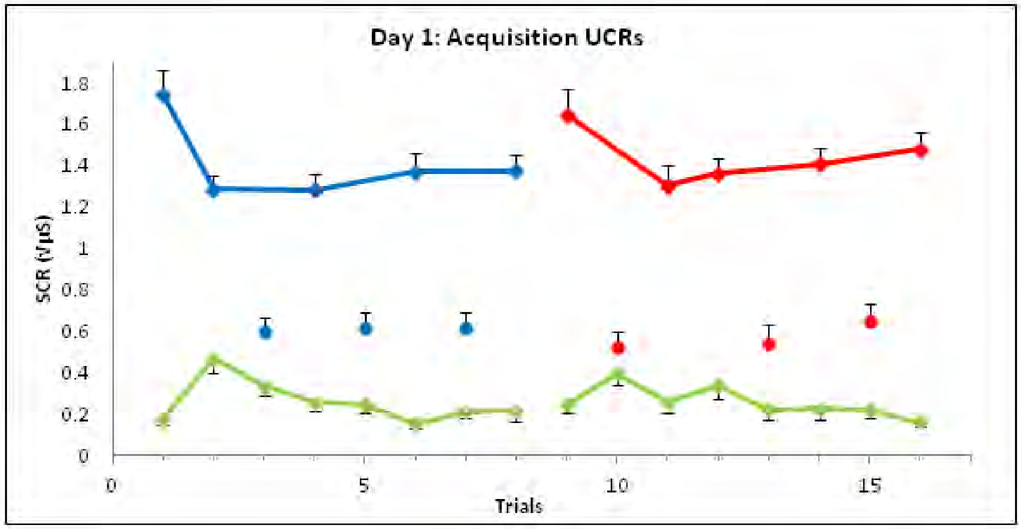

Figure 6. Group mean skin conductance responses during US interval following CS+R, CS+N, and CS- trials for the Acquisition phase (day 1). Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Unconnected circles represent unreinforced (i.e., no shock) CS+ trials. SCR = skin conductance response.

STUDY 3: MIFEPRISTONE INTERVENTION

Mifepristone Reduces Skin Conductance Reactivity to Fear-Conditioned Stimuli

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Abstract

Mifepristone was examined for its ability to impair the reconsolidation of conditioned fear as measured by skin conductance responses (SCRs), as a step towards reducing fearfulness in anxiety disorders and posttraumatic stress disorder. The present study used videos of biologically prepared, conditioned stimuli (tarantulas) in healthy young adults. Strong differential conditioning, as measured by SCR, was observed among a screened subset of participants during acquisition. When adjusted for the strength of the conditioned response during acquisition, a single presentation of the conditioned stimulus after administering mifepristone was found to reduce the conditioned SCR to the fear stimulus when tested a day later. These results suggest that mifepristone has the potential to reduce reconsolidation of a conditioned fear memory.

Mifepristone Reduces Skin Conductance Reactivity
to Fear-Conditioned Stimuli

Basic research has demonstrated that reactivation of a consolidated memory may return it to an unstable state from which it must be restabilized (i.e. reconsolidated) in order to persist (Debiec & Ledoux, 2004; Nader, Schafe, & Le Doux, 2000; Nader & Einarsson, 2010). Reconsolidation is governed by neurobiological processes similar to those of consolidation (Lee, Everitt, & Thomas, 2004), and it is susceptible to pharmacological blockade (Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Pitman et al., 2011; Przybyslawski, Roullet, & Sara, 1999). Reactivation of a persistent trauma-related memory and subsequent interference with reconsolidation of the memory may offer a novel treatment strategy for posttraumatic stress disorder (PTSD).

Mifepristone (RU-486) is a pharmacological agent that has offered promise as a reconsolidation blocker. It is widely and safely used to induce abortion due to its anti-progestin effects, but it is also a powerful glucocorticoid receptor antagonist. Reactivated fear memories have been shown to be sensitive to mifepristone in animals (Jin et al. 2007; Taubenfeld et al. 2009; Pitman et al. 2011). However, in two studies of individuals with PTSD, Wood et al. (2014) reported that mifepristone failed to block reconsolidation of trauma-related memories. These negative findings may have been attributable to relatively low PTSD severity in the samples studied, creating a floor effect that did not allow potential drug effects to be detected. It is possible that a fear conditioning procedure that is capable of optimizing the strength of the conditioned fear response, might provide a more sensitive test of mifepristone's ability to block memory reconsolidation in humans.

We have created a Pavlovian fear conditioning paradigm that can be used to test the relative strengths of various drug and non-drug candidates for reconsolidation blockade (Spring, Wood, Mueller-Pfeiffer, Milad, Pitman & Orr, 2015). To this end, we have used more highly prepared CSs, more fear-sensitive subjects, and stronger conditioned responses (CRs). First, it has been shown that certain classes of CSs, when paired with a US, produce a stronger fear CR, i.e., they are more "prepared" to enter into an association with the US (Mineka & Öhman, 2002). We enhanced preparedness of the CSs by using 12-sec, high-definition video clips of three crawling tarantulas, each different in appearance. Second, we limited enrollment to participants who scored approximately one standard deviation or greater above the population mean on the Spider Phobia Questionnaire-15 (SPQ-15), as described by Olatunji et al. (2009); however, anyone who endorsed symptoms of clinical spider phobia was excluded. Third, we required that participants show evidence of strong differential conditioning, as determined by a more stringent cutoff assigned to the CRs recorded during Day 1 acquisition (specified below). Participants with subthreshold CRs were withdrawn after Day 1.

This procedure was previously used to test the whether or not propranolol could reduce reconsolidation of a fear-conditioned skin conductance response (SCR; Spring et al., 2015). Spring et al. found that the differential conditioning procedure, combined with setting a differential conditioning threshold for acquired SCRs, produced robust responses to the stimuli that could subsequently serve as suitable conditioned stimuli to be reactivated (CS+R) or not (CS+N), by which reconsolidation blockade of conditioned fear responses could be assessed. On the day following fear conditioning, reconsolidation blockade was attempted by administering

propranolol followed by a single presentation of one of the two CS+s (i.e., the CS+R). Unfortunately, Spring et al. found that propranolol had no measurable reconsolidation-blocking effect on the fear-conditioned SCR. Interestingly, an earlier study by Soeter and Kindt (2010) found that propranolol effectively blocked reconsolidation of conditioned fear as measured by the eyeblink startle response, but not the SC response.

The present study aimed to test the efficacy of a new drug, viz., mifepristone, in blocking reconsolidation and thereby reducing the fear memory within the same conditioning paradigm. We hypothesized that mifepristone would reduce the SCR to a previously conditioned stimulus when this stimulus was reactivated (CS+R) in the presence of the drug, compared to a previously conditioned stimulus that was not reactivated (CS+N). A within-subjects design was employed; there was no placebo condition.

Method

Participants

The study protocol was approved by the Partners Human Research Committee (PHRC), as well as the United States Army Medical Research and Material Command (USAMRMC) Human Research Protection Office (HRPO). After a full explanation of the procedures, all participants provided written informed consent.

Prior to enrollment, participants were screened by phone to verify the presence of a manageable, non-phobic fear of spiders as determined by scores above the mean on the SPQ-15 (Olatunji et al., 2009), but the absence of specific spider phobia criteria according to the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-IV; First, Spitzer, Gibbon, & Williams, 1997). Participants underwent a set of screening criteria taken directly from the SCID-IV to verify absence of other current mental disorders including substance abuse. Subjects with serious potentially complicating medical or neurological conditions were also excluded. A urine drug screen verified the absence of illicit substances and psychotropic medications.

Forty-four healthy participants (26 females, 18 males) were enrolled in the study. Of these, none had an unmeasurable (very low) SC level or a current Axis I psychiatric disorder; however, 13 dropped out prior to beginning the Day 1 procedure. Following the Day 1 procedure, 18 were withdrawn due to failure to demonstrate adequate differential conditioning (conditioning criteria are described below in Data Reduction). The remaining 13 (7F, 6M) who underwent study procedures on Days 1-3 had a mean age of 23.2 years (SD = 4.5, range 18 to 34 years) and a mean score on the SPQ-15 of 8.0 (SD = 2.5, range 4.5 to 14 of a possible 0 to 15). Mean years of education was 15.2 (SD = 1.6, range 13 to 17 years). Five of the 13 participants were not included in the Day 30 analyses due to being lost to follow-up.

Equipment and Stimuli

Equipment and stimuli were the same as those used by Spring et al. (2015). Skin conductance analog signals were recorded using a Coulbourn Lab Linc V Series Human Measurement System (Coulbourn Instruments, Whitehall, PA) with a Coulbourn Isolated Skin Conductance Coupler (V71-23) through 8mm (sensor diameter) Ag/AgCl electrodes (In Vivo Metric; Healdsburg, CA) filled with an isotonic paste. Electrodes were separated by 14mm, as determined by the width of the adhesive collar, and placed on the hypothenar surface of the subject's non-dominant hand in accordance with published guidelines (Boucsein et al., 2012; Fowles et al., 1981). The SC signal

was sampled at 1000 Hz and digitized by a Coulbourn Analog to Digital Converter (V19-16). A Cobalt notebook computer (IBM-compatible; Cobalt Computers, Whitehall, PA) with custom-designed software was used to record and store the digitized physiological signals.

Nine high-definition video clips (Virtually Better Inc., Decatur, GA) depicting one of three tarantulas occupying one of three contexts comprised the conditioned stimuli (CS). Two of the three tarantulas always served as the CS+s, either the to-be-reactivated CS+ (CS+R) or the to-be-non-reactivated CS+ (CS+N), and were paired with the unconditioned stimulus (US, shock) on Day 1. The to-be CS+R served as the stimulus that was presented on Day 2 after the study medication was administered; the to-be CS+N was not presented on Day 2. The third tarantula served as the CS- and was not paired with the US on Day 1 and also not presented on Day 2. The three contexts within which the tarantulas appeared were a virtual kitchen (A), bedroom (B) and office (C). The particular tarantula that served as the CS+N or CS+R was counterbalanced across participants; the tarantula used to represent the CS- was the same across subjects.

The US was a 0.5-sec mild electric shock that ranged in intensity from 0.2 to 4.0 milliamperes according to the level determined by the participant. The US was delivered using a Coulbourn Transcutaneous Aversive Finger Stimulator (E13-22) through shock electrodes attached to the middle segments of the 2nd and 3rd fingers on the hand opposite to that on which the SC recording electrodes were attached.

The video clips lasted 12 seconds each: four seconds of context alone (i.e., no tarantula), followed by eight seconds of context plus tarantula. On reinforced trials, the US immediately followed the CS+ offset. The intertrial interval consisted of a black screen and was randomized to last 16, 18, 20, 22, or 24 seconds. The procedure was implemented using E-Prime Professional 2.0 (Psychology Software Tools, Inc., Sharpsburg, PA).

Procedure

As depicted in Figure 7, the procedure consisted of a differential fear-conditioning paradigm that entailed laboratory visits over three consecutive days and a one-month follow-up visit (M = 27.75 days; SD = 0.46). On day 1, participants were instructed: *"Today, you will be viewing videos of spiders on the television, and you will receive electric shocks on your fingers after viewing some of the spiders. These shocks will be annoying, but not painful. We will also use electrodes on your palm to record how your body responds to this procedure."* Following these instructions, participants selected their own shock to a level to be “highly annoying but not painful” (Orr et al., 2000). Participants were then shown still images, i.e., screenshots, of the three tarantulas that would serve as CSs, accompanied by these instructions: *"During the experiment, it will be important that you are able to tell these spiders apart. To do this, try focusing on the legs. For this spider, note the alternating black and white stripe pattern. For this spider, note the orange highlights. For this spider, note that the legs are solid black."* Prior to beginning the procedure, the lights were dimmed and over-ear headphones placed on the participant to reduce ambient noise and enable communication with study staff in the next room. Participants were instructed to sit still in the chair, keep their eyes open, and be attentive to the stimuli presented on the screen. Next, there was a 5-min baseline period to record physiological levels.

Day 1 consisted of two sequential phases: 1) unreinforced presentations of each of the nine possible spider-context combinations in pseudorandom order (*habituation*), and 2) eight partially reinforced (i.e., five of eight) presentations each of CS+R and CS+N, presented separately in blocks and interspersed pseudorandomly with eight presentations of CS- (*acquisition*). Presentation order of CS+R and CS+N trial blocks was counterbalanced across participants. All CS+R, CS+N, and CS- presentations during acquisition occurred within context A. Participants who did not meet the defined cutoff for demonstrating a differential conditioned response (see below) were withdrawn prior to Day 2.

The procedures for Days 2, 3, and the 1-month follow-up were largely the same as for Day 1, with the following exceptions: a) the procedure for setting the level of shock was not repeated, as the shock level determined on the first visit was used for the remainder of the study, b) participants were only familiarized with images of the stimuli prior to undergoing the Day 1 procedure, and c) rather than “will receive” as on Day 1, participants were instructed that they “may or may not receive” electric shocks.

Day 2 consisted of the participant receiving an 1800 mg dose of mifepristone (Danco Laboratories, LLC, New York, NY), which was followed 90 minutes later by a single, unreinforced presentation of the CS+R (*reactivation*). Reactivation of the CS+R occurred in context B. Following oral administration, mifepristone reaches a peak plasma concentration after approximately 90 minutes. In a pre-clinical animal study, reconsolidation blockade was found with a dose of 30 mg/kg, which on a kg-for-kg basis corresponds to approximately 1800 mg in a 60 kg human (Pitman et al. 2011). The 1800 mg mifepristone dose employed here represents the maximum that has been approved for use in the USA (albeit for a different indication).

Day 3 consisted of three sequential phases: 1) two unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*renewal test*); and 2) three unsignalled presentations of the US alone, followed by 3) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*reinstatement test trials* and *extinction*). All presentations of the CSs occurred in context A. Ordering of CS+R and CS+N presentations, within the full set of trials that included CS- presentations, was counterbalanced across subjects.

The one-month follow-up consisted of two phases: 1) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*spontaneous recovery test* and *re-extinction*), followed by 2) eight partially-reinforced, i.e., five of eight, presentations each of CS+R and CS+N presented in successive blocks and interspersed with eight CS- trials for the respective blocks as was done on day 1 (*re-acquisition/savings test*). All stimuli were presented in context C during this visit; the CS+R block of trials was presented first.

Physiological Measures and Data Reduction

As previously described (Milad, Orr, Pitman, & Rauch, 2005; Orr et al., 2000), an SCR for the CS interval was calculated for each trial by subtracting the mean SC level during the two sec prior to CS onset (context alone presentation) from the peak SC level during the eight sec CS interval. These SCR values reflect change in skin conductance level beyond that resulting from presentation of context alone. A square root transformation was applied to the absolute value of each SCR, followed by replacement of the + or - sign, prior to statistical analysis.

For day 1, the untransformed SCR data were scored to determine whether a definable differential SCR was obtained for *both* the CS+R and CS+N during the acquisition phase. We

averaged SCRs across respective CS+R, CS+N, and CS- trials in order to calculate a difference score between the CS+R and its respective CS- trials and between the CS+N and its respective CS- trials. A cutoff of .1µS was applied to each difference score and participants with one or both difference scores below this cutoff were withdrawn from the study prior to Day 2.

Data Analytic Strategy

The primary statistical approach was a mixed-model, repeated measures analysis of variance (ANOVA), performed separately for each experimental phase. Participants were treated as a random effect, Stimulus (CS+R, CS+N, CS-) as a within-participants effect, and Trials as the repeated measure. The criterion for statistical significance was $p<0.05$, two-tailed.

Results

Acquisition Phase (day 1).

Responses to the CSs during the acquisition phase were examined across the eight presentations of each CS using a mixed-model, repeated-measures ANOVA with two factors: Stimulus (CS+R, CS- or CS+N, CS-) and Trials. As can be seen in Table 3 and Figure 8, top panel, during the acquisition phase, the CS+R and CS+N produced larger SCRs, compared to their respective CS- trials. There was a trend for somewhat larger magnitude SCRs to the CS+R, compared to CS+N, trials. There were significant Stimulus x Trials interactions for the comparisons of CS+R to CS- ($F(7, 84) = 2.18$, $p = .044$, $Eta^2 = .02$) and CS+R to CS+N ($F(7, 84) = 3.14$, $p = .005$, $Eta^2 = .06$).

SC responses for the US interval, which represented the unconditioned response (UR), were calculated and plotted for the acquisition phase (see Figure 9). Because SCR onset has a known latency of 1-2 s (Edelberg, 1967), the 1-s interval immediately following US onset was used as the baseline for calculating the SC UR, which was subtracted from the peak SC level within the 6-s interval following US onset to yield the UR. A square root transformation was applied to the UR, as was done for the CR. As expected, both CS+R and CS+N trials produced larger SCRs during the US interval, compared to CS- trials ($F(1, 13) = 87.80$, $p < .001$, $Eta^2 = .38$; $F(1, 13) = 137.27$, $p < .001$, $Eta^2 = .40$; respectively). There were significant Stimulus x Trials interactions for both CS+R and CS+N trials ($F(7, 84) = 12.99$, $p < .001$, $Eta^2 = .13$; $F(7, 84) = 18.71$, $p < .001$, $Eta^2 = .18$; respectively). The average UR magnitudes for CS+R and CS+N reinforced trials did not differ ($F(1, 13) < 1$, p = NS). There was a significant Stimulus x Trials interaction for the comparisons of CS+R to CS+N trials ($F(7, 84) = 12.16$, $p < .001$, $Eta^2 = .11$).

Renewal Phase (Day 3).

Responses to the CSs during the renewal phase were examined over the two presentations of each CS using a two-factor (Stimulus, Trials) repeated-measures ANOVA. The Stimulus factor had two levels in each of three ANOVAs that compared CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials; the Trials factor has 2 levels. As can be seen in Table 3 and Figure 8, middle panel, SCRs to the CS+N were significantly larger than to the CS-, demonstrating a persistence of the conditioned fear response; whereas, SCRs to the CS+R were not significantly larger than to the CS-. The Stimulus x Trials interaction effect was significant for the comparison of CS+R to CS- trials ($F(7, 84) = 10.40$, $p = .007$, $Eta^2 = .05$) and nearly significant for the comparison of CS+N to CS- trials ($F(7, 84) = 4.64$, $p = .052$, $Eta^2 = .01$).

SCR magnitudes to the CS+R and CS+N were not significantly different when they were directly compared. However, as can be seen in Figure 8, top panel, and as suggested by the pattern of ANOVA results for the Day 1 Acquisition Phase, there appeared to be stronger differential conditioning to the CS+R than to the CS+N. In order to adjust for possible differences in strengths of the conditioned responses to the CS+R and CS+N, averaged SCRs were calculated for the two CS+R and two CS+N Renewal Phase trials and divided by their respective difference scores for the Day 1 acquisition trials. The Acquisition Phase difference scores were calculated by averaging the SCRs to the eight CS+R trials and subtracting the average SCR for the respective eight CS- trials and by averaging the SCRs to the 8 CS+N trials and subtracting the average SCR for the respective eight CS- trials. A comparison of the adjusted CS+R (M = 1.75 μS, SD = 1.05) and adjusted CS+N (M = 3.03 μS, SD = 1.94) indicated that the adjusted response to the CS+R was significantly smaller than the adjusted response to the CS+N ($F(1, 12) = 7.47$, $p = .018$, Cohen's $d = .82$).

Reinstatement Phase (Day 3).

Responses to the CSs during the reinstatement and extinction phase, which immediately followed the unsignalled shock presentations, were first examined across the ten presentations of each CS using a two-factor (Stimulus, Trials), repeated measures model. The Stimulus factor has three levels (CS+R, CS+N, CS-); the Trials factor has ten levels. The main effect of Stimulus ($F(2, 24) = 1.30$, $p = .29$, $Eta^2 = .01$) and the Stimulus x Trials interaction ($F(18, 216) < 1$, $p =$ NS) were not significant. For the interested reader, direct comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials are provided in Table 3. As can be seen in Table 3, the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N SCR magnitudes did not yield significant differences. The Stimulus x Trials interaction effects were also not significant for the comparisons of CS+R to CS- trials ($F(9, 108) < 1$, $p =$ NS), CS+N to CS- trials ($F(9, 108) = 1.24$, $p = .28$, $Eta^2 = .03$) and CS+R to CS+N trials ($F(9, 108) < 1$, $p =$ NS).

In order to avoid the potential dampening of differences in SCR to the CSs due to rapid extinction, we also performed two-factor (Stimulus, Trials), mixed-model repeated measures ANOVA that only considered the first two presentations of each CS during the reinstatement phase. For these analyses, the Stimulus factor has two levels in the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials and the Trials factor has two levels. As can be seen in Table 3, SCR magnitudes to the CS+N showed a nearly significant trend towards being larger than those to the CS-, whereas there was no difference when comparing CS+R and CS- trials. However, there was no difference between SCR magnitudes to the CS+R and CS+N presentations when they were directly compared. As was done for the Renewal Phase, in order to adjust for possible differences in strengths of the conditioned responses to the CS+R and CS+N, averaged SCRs were calculated for the two CS+R and 2 CS+N Renewal Phase trials and divided by their respective difference scores for the day 1 acquisition trials. A comparison of the adjusted CS+R (M = 1.04 μS, SD = 0.74) and adjusted CS+N (M = 1.54 μS, SD = 0.90) indicated that the adjusted response to the CS+R was significantly smaller than the adjusted response to the CS+N ($F(1, 12) = 4.94$, $p = .046$, Cohen's $d = .61$).

Re-extinction Phase (1 Month).

Spontaneous recovery of the conditioned SCR was assessed by examining response magnitudes of the first two CS+R, CS+N and CS- trials during the re-extinction phase using mixed-model, two-factor (Stimulus, Trials) repeated measures ANOVAs; the Stimulus factor has

3 levels (CS+R, CS+N, CS-) and the Trials factor has two levels. The Stimulus main effect ($F(2, 14) < 1$, p = NS) and Stimulus x Trials interaction ($F(2, 14) < 1$, p = NS) were not significant. Direct comparisons between SCR magnitudes of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials were made using two-factor (Stimulus, Trials), mixed model repeated measures ANOVA. For these analyses, the Stimulus factor has two levels in the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials and the Trials factor has two levels. As can be seen in Table 3, none of these direct comparisons yielded significant differences. When SCRs were examined across all re-extinction trials, there was no significant Stimulus main effect ($F(2, 14) < 1$, p = NS), as well as no significant Stimulus x Trials interaction ($F(18, 126) < 1$, p = NS) (Figure 8, bottom panel).

Re-acquisition Phase (1 Month).

Responses to the CSs during the re-acquisition phase were examined across the eight presentations of each CS using a two-factor (Stimulus, Trials) repeated measures mixed-model with the Stimulus factor having two levels for each of 3 ANOVAs comparing CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials and the Trials factor with eight levels. As can be seen in Table 3 and Figure 8, bottom panel, we observed a pattern somewhat similar to that for acquisition on day 1. Reactivity to CS+R and CS+N was stronger when compared to their respective CS- trials, but there was no significant difference in the magnitudes of SCRs to the CS+R and CS+N. The Stimulus x Trials interaction was not significant when comparing CS+R to CS- ($F(7, 49) = 1.42$, $p = .22$, $Eta^2 = .03$) and CS+R to CS+N ($F(7, 49) < 1$, p = NS), and marginally significant when comparing CS+N to CS- ($F(7, 49) = 2.06$, $p = .07$, $Eta^2 = .06$).

Discussion

Brief clips of moving tarantulas depicted within various contexts served as the conditioned stimuli for fear-conditioned SCRs. As previously observed by Spring et al. (2015), the differential conditioning procedure, combined with setting a differential conditioning threshold for SCRs, produced robust responses to the conditioned stimuli that would subsequently serve as the reactivated (CS+R) and non-reactivated (CS+N) cues, by which reconsolidation blockade of conditioned fear responses was assessed. On the day following fear conditioning, reconsolidation blockade of the CR was attempted by administering mifepristone followed 90 min later by a single reactivation presentation of one of the two CS+s (CS+R). On subsequent testing the next day (Renewal Phase), we found evidence for a measurable reconsolidation-blocking effect on the fear-conditioned SCR to the CS+R. Presentation of the non-reactivated CS+ (CS+N) produced significantly larger SCRs, compared to SCRs to the CS, whereas SCRs to the CS+R did not differ significantly from those to the CS-. Because differential conditioning appeared to be stronger to the CS+R than to the CS+N during acquisition, the comparison between responses to the CS+R and CS+N during the Renewal Phase test trials and the first two trials of the Reinstatement Phase were adjusted for the differential responses during acquisition. These comparisons indicated that there were proportionately smaller responses to the CS+R than to the CS+N, suggesting that mifepristone was effective in reducing reconsolidation of the fear response to the reactivated CS+.

The present findings for mifepristone are encouraging and contrast with the negative findings for propranolol reported by Spring et al. (2015), which used the same conditioning procedure as used in the present study. The studies of Soeter and Kindt (2010, 2011) found that propranolol blocked reconsolidation of a conditioned fear response when the fear was assessed using a

potentiated startle response, but not when measured with SCR (Soeter & Kindt, 2011). The present findings indicate that a fear conditioned SCR can be used as an index of reconsolidation blockade. Furthermore, the protocol used by Soeter and Kindt (2010) produced a floor effect, wherein there was a 100% reduction of the fear memory trace, as measured by fear-potentiated startle, in the propranolol-reactivation condition. Because of this floor effect, the protocol would not be able to detect an intervention that is potentially more effective than propranolol. The amount of reconsolidation blockade produced by mifepristone and evidenced by the reduced SCR using the present procedure was sufficiently modest so as to allow for the detection of more effective drugs or interventions.

Spring et al. (2015) noted that the effort to develop a protocol that generated stronger differential conditioning that would be more resistant to a floor effect (i.e., use of an SPQ-15 cutoff score, more salient stimuli, and a stringent conditioning criterion) may have produced conditioning that was highly resistant to noradrenergic blockade, possibly explaining their negative finding for propranolol. If correct, it would seem that mifepristone may be a worthy candidate intervention for a future clinical trial. The positive effect of mifepristone on reconsolidation blockade observed in the present study stands in contrast to the negative findings from the two clinical-sample studies reported by Wood et al. (215). It is possible that these negative results reflect mifepristone's potentially poor efficacy for blocking reconsolidation of traumatic memories. However, as noted by Wood et al., it is possible that the script-driven imagery procedure used in the studies may sometimes be insufficient to induce traumatic memory destabilization (see Sevenster et al. 2012; Soeter & Kindt, 2013). Also, as pointed out earlier, those negative findings may be attributable to the clinical samples' relatively low PTSD severity, which did not allow for the potential effect of mifepristone to be detected.

An important limitation of the present study rests with the difference in the strengths of the conditioned SCRs to the CS+R and CS+N. This difference necessitated that adjustments be made to the SCRs during the Renewal phase, which provided the primary test of whether mifepristone successfully reduced reconsolidation of the fear memory. Thus, the efficacy of mifepristone was only evident in comparisons of the adjusted SCRs for the CS+R and CS+N. It is possible that the significant differences observed in our study are a consequence of the adjustment and may not be reliable. The larger conditioned SCRs to the CS+R, compared to CS+N, during acquisition are likely due to chance and the smaller sample size of the present study. In our previous work, we have found there to be comparably strong conditioned responses to the CS+R and CS+N (Spring et al., 2015; Frichionne et al., in preparation). There are a number of potential side-effects associated with mifepristone, and a number of individuals declined to participate in the present study because of these possible side-effects. This is also likely responsible for the seemingly high pre-Day1 dropout rate. Although, these possible side-effects are unlikely when taking taking mifepristone and none of our participants experienced any, the potential for an adverse consequence/reaction may limit some patients' willingness to use it.

Table 3: Results of mixed-model repeated measures ANOVA of SCR (√μS) for the CS interval.

			DF	F	p	Eta²
Day 1	Acquisition	CS+R vs CS-	1, 12	109.49	<.001	0.31
		CS+N vs CS-		45.13	<.001	0.22
		CS+R vs CS+N		4.34	.06	0.04
Day 3	Renewal	CS+R vs CS-	1, 12	3.40	.09	0.08
		CS+N vs CS-		18.93	<.001	0.15
		CS+R vs CS+N		1.03	0.33	0.03
	Reinstatement (2 trials)	CS+R vs CS-	1, 12	2.40	0.15	0.04
		CS+N vs CS-		4.02	0.07	0.08
		CS+R vs CS+N		<1	NS	0.00
	Reinstatement and Extinction (10 trials)	CS+R vs CS-	1, 12	1.75	0.21	0.01
		CS+N vs CS-		1.75	0.21	0.01
		CS+R vs CS+N		<1	NS	0.00
1 Month	Re-extinction Spontaneous recovery (2 trials)	CS+R vsCS-	1,7	<1	NS	0.00
		CS+N vs CS-		<1	NS	0.00
		CS+R vs CS+N		<1	NS	0.01
	Re-acquisition	CS+R vs CS-	1,7	16.01	0.005	0.13
		CS+N vs CS-		14.43	0.007	0.12
		CS+R vs CS+N		<1	NS	0.01

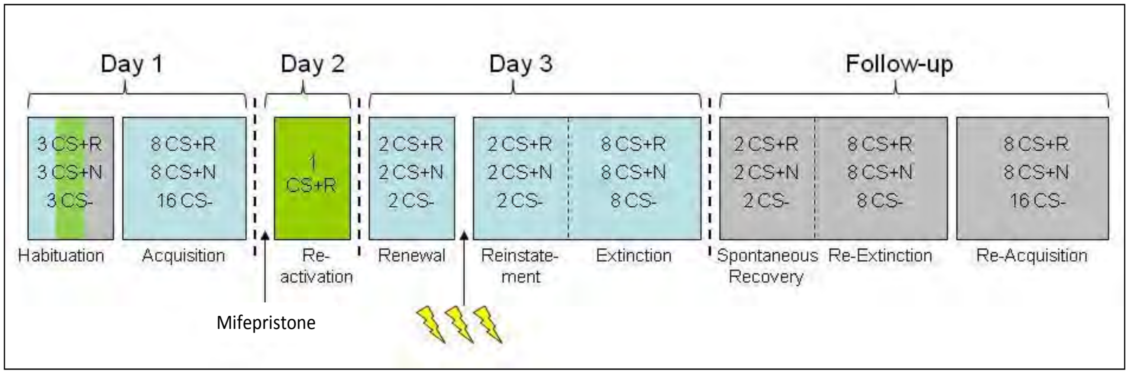

Figure 7: Depiction of the 4-session fear-conditioning procedure. Fear conditioning occurred on day 1. A single dose of 1800 mg of mifepristone was administered on day 2, followed by reactivation of one of the two the CS+s (i.e., the CS+R). On day 3 and the 1-month follow-up visit, post-intervention reactivity to the conditioned stimuli was tested. CS+R = CS+ to-be-reactivated; CS+N = CS+ non-reactivated; CS- = unreinforced. Lightning bolts represent unsignalled presentations of the US alone. Shading colors represent the context in which the stimuli were presented: blue = A, green = B, grey = C. CS+R and CS+N acquisition and re-acquisition trials occurred in blocks as described in the text.

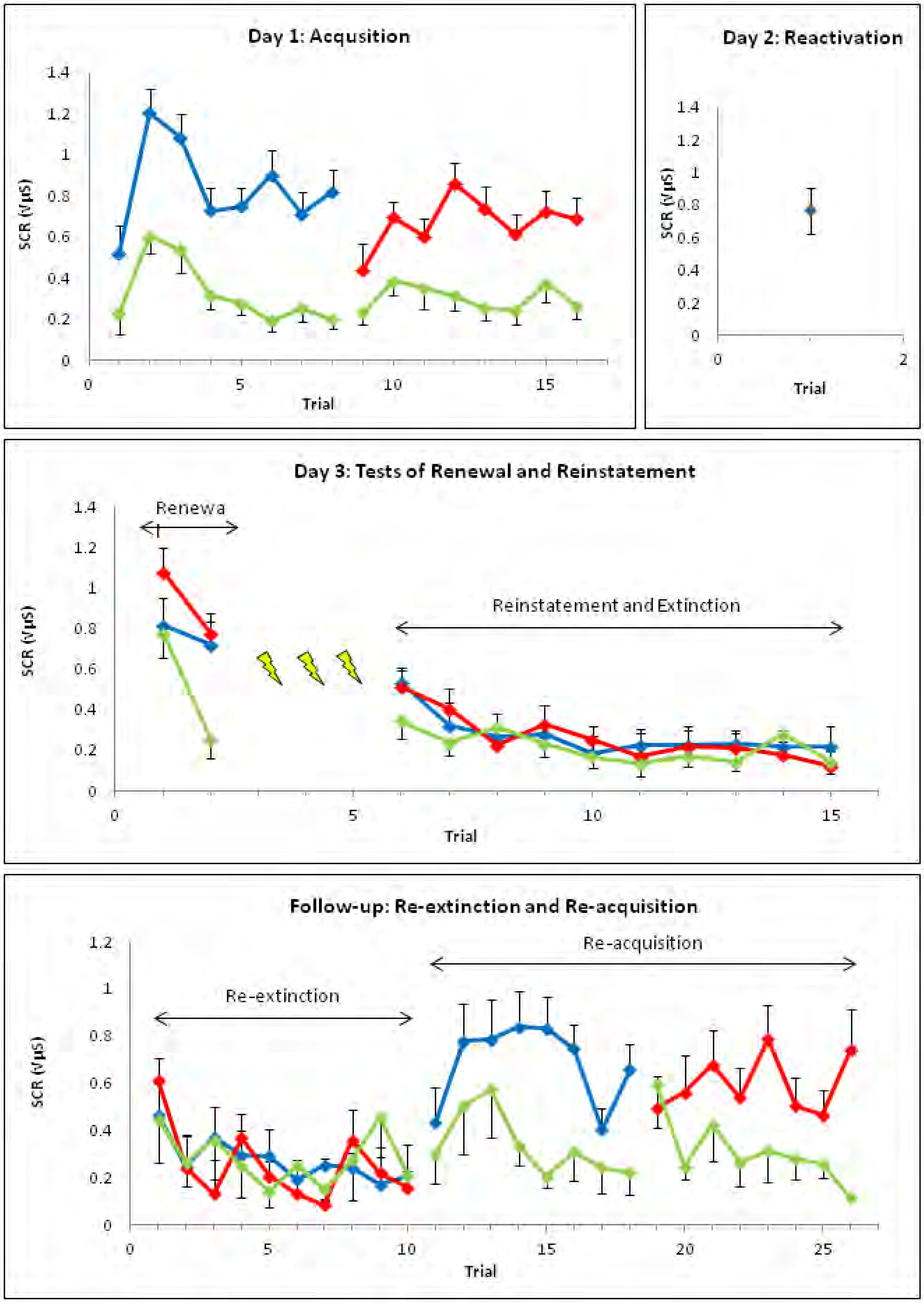

Figure 8. Group mean skin conductance responses to CS+R, CS+N, and CS- trials for the Acquisition (day 1), Reactivation (day 2), Renewal and Reinstatement (day 3), and Re-extinction and Re-acquisition (day 30) phases. Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Lightning bolts represent unsignaled presentations of the US alone. SCR = skin conductance response.

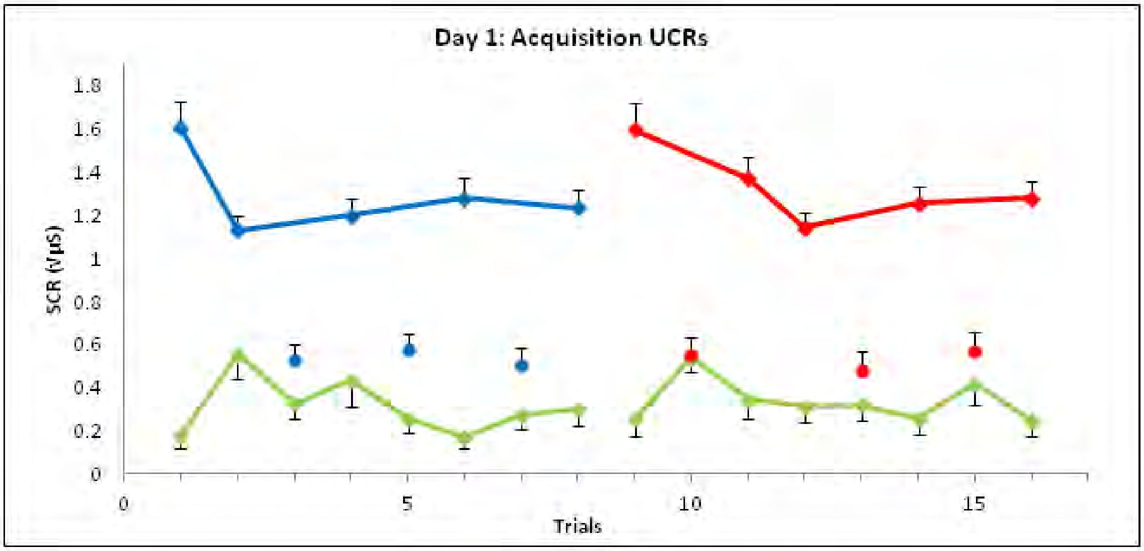

Figure 9. Group mean skin conductance responses during US interval following CS+R, CS+N, and CS- trials for the Acquisition phase (day 1). Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Unconnected circles represent unreinforced (i.e., no shock) CS+ trials. SCR = skin conductance response.

STUDY 4: OXYTOCIN INTERVENTION

Oxytocin May Produce a Generalized Reduction of Skin Conductance Reactivity to Fear-Conditioned Stimuli

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Abstract

Oxytocin was examined for its ability to impair memory reconsolidation of fear-conditioned skin conductance (SC) responses as a step towards providing a means for reducing fearfulness in anxiety disorders and posttraumatic stress disorder. The present study used videos of biologically prepared, conditioned stimuli (CSs, tarantulas) to test the efficacy of intranasal oxytocin in reducing reconsolidation of conditioned fear in healthy young adults. Strong differential conditioning to two CSs, as measured by SC, was observed among a screened subset of participants during acquisition. On the day following fear conditioning, one of the two CSs was presented once after the participant received intranasal oxytocin. When tested the following day, conditioned SC responses to the reactivated and non-reactivated CS were both reduced and did not differ from each other. The small sample size (n=5) precludes firm conclusions. However, the possibility is raised that oxytocin's influence on reconsolidation of a conditioned fear memory may generalize from a reactivated CS to a non-reactivated CS.

Oxytocin May Produce a Generalized Reduction of Skin Conductance Reactivity to Fear-Conditioned Stimuli

Basic research has demonstrated that reactivation of a consolidated memory returns it to a destabilized state from which it must be restabilized (i.e. reconsolidated) in order to persist (Debiec & Ledoux, 2004; Nader, Schafe, & Le Doux, 2000; Nader & Einarsson, 2010). Reconsolidation is governed by neurobiological processes similar to those of consolidation (Lee, Everitt, & Thomas, 2004), and is susceptible to pharmacological blockade (Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Pitman et al., 2011; Przybyslawski, Roullet, & Sara, 1999). Reactivation of a persistent trauma-related memory and subsequent interference with *reconsolidation* of the memory may offer a feasible treatment strategy for posttraumatic stress disorder (PTSD).

Animal research going back nearly fifty years has shown that the posterior pituitary hormone oxytocin has a biphasic effect on memory in rodents. First, it reduces the consolidation of new fear learning. Second, it reduces the retrieval of learned (consolidated) information. Also, oxytocin has been found to inhibit activation in the amygdala, which could potentially interfere with reconsolidation of fear memory (Huber, Veinante & Stoop, 2005). Pitman and colleagues (unpublished) have evaluated the ability of several drugs, including oxytocin, given immediately following reactivation of a conditioned fear memory to reduce subsequent conditioned responding in rats, presumably through blockade of memory reconsolidation. The procedure included 3 days of testing. On Day 1, a 30-s tone conditioned stimulus (CS) was paired with an electric shock unconditioned stimulus (US). On Day 2, the CS was presented without the US (reactivation), and the freezing conditioned response (CR) was measured. This was immediately followed by drug. On Day 3, the CR was again measured as a test. Data for oxytocin were obtained using a 0.75 mV unconditioned stimulus (UCS), with n=24 at each of three dosage levels: 0.05, 1.25, and 10 mg/kg.

During the first oxytocin trial (A), postreactivation oxytocin at dosages of 1.25 mg/kg and 10 mg/kg, but not at a dosage of 0.05 mg/kg, given on Day 2 significantly reduced conditioned fear responding in comparison to vehicle (placebo) on Day 3. In a second oxytocin trial (B), postreactivation oxytocin at a dosage of 1.0 mg/kg reduced subsequent conditioned responding to a greater degree than vehicle, but the results did not achieve statistical significance. The decrease in percent freezing (i.e, decrease in the conditioned response or CR) from Day 2 (i.e., freezing to the CS measured immediately before drug is given) to Day 3 (i.e., freezing measured one day after reactivation+drug) was measured in the rats. This decrease is a putative index of the degree of reconsolidation blockade induced by the drug. Effect sizes calculated as the change score for each drug trial minus the change score for the pooled vehicle (VEH) trials, divided by the SD of the latter, indicated that oxytocin at dosages of 1.25 and 10 mg/kg met the cut-off for statistical significance at $p<0.05$.

Research on the effect of oxytocin on memory in humans has been limited. Pitman, Orr and Lasko (1993) measured heart rate, skin conductance, and lateral frontalis electromyographic (EMG) responses in 43 male Vietnam veterans with posttraumatic stress disorder during personal combat imagery. In a double-blind research design, subjects were randomly assigned to receive intranasal arginine vasopressin (20 IU), placebo, or oxytocin (20 IU) an hour before the experiment. The group order of physiologic responding was as predicted: vasopressin > placebo

> oxytocin. This drug effect was not accounted for by nonspecific changes in responsiveness. These results are consistent with enhancing and inhibiting effects on memory retrieval and conditioned responding of vasopressin and oxytocin, respectively. The results support the conclusion that intranasally administered oxytocin in a dosage of 20 IU is capable of affecting memory in humans, although the results pertained to memory retrieval and not to consolidation or reconsolidation. Several recent research studies have found that intranasal oxytocin is capable of exerting effects on human cognition and behavior (e.g., Di Simplicio, Massey-Chase, Cowen & Harmer, 2009; Heinrichs, Meinlschmidt, Wippich, Ehlert & Hellhammer, 2004). In light of the promising animal and human results with oxytocin-induced reconsolidation blockade discussed above, we believe an investigation in normal humans is warranted.

We have created a Pavlovian fear conditioning paradigm that can be used to test the relative strengths of various drug and non-drug candidates for reconsolidation blockade (Spring, Wood, Mueller-Pfeiffer, Milad, Pitman & Orr, 2015). To this end, we have used more highly prepared CSs, more fear-sensitive subjects, and stronger conditioned responses (CRs). First, it has been shown that certain classes of CSs, when paired with a US, produce a stronger fear CR, i.e., they are more “prepared” to enter into an association with the US (Mineka & Öhman, 2002). We enhanced preparedness of the CSs by using 12-sec, high-definition video clips of three crawling tarantulas, each different in appearance. Second, we limited enrollment to participants who scored approximately one standard deviation or greater above the population mean on the Spider Phobia Questionnaire-15 (SPQ-15), as described by Olatunji et al. (2009); however, anyone who endorsed symptoms of clinical spider phobia was excluded. Third, we required that participants show evidence of strong differential conditioning, as determined by a more stringent cutoff assigned to the CRs recorded during Day 1 acquisition (specified below). Participants with subthreshold CRs were withdrawn after Day 1.

This procedure was previously used to test the whether or not propranolol could reduce reconsolidation of a fear-conditioned skin conductance (SC) response (Spring et al., 2015). Spring et al. found that the differential conditioning procedure, combined with setting a differential conditioning threshold for SCRs, produced robust responses to the stimuli that would subsequently serve as the conditioned stimuli to be reactivated (CS+R) and non-reactivated (CS+N), by which reconsolidation blockade of conditioned fear responses was assessed. On the day following fear conditioning, reconsolidation blockade was attempted by administering propranolol followed by a single presentation of one of the two CS+s (i.e., the CS+R). Spring et al. found that propranolol had no measurable reconsolidation-blocking effect on the fear-conditioned SC response. Interestingly, an earlier study by Soeter and Kindt (2010) found that propranolol effectively blocked reconsolidation of conditioned fear as measured by reduction of the fear-potentiated startle (eyeblink response), but not the SC response.

The present study aimed to test the efficacy of oxytocin in blocking reconsolidation and thereby reducing the fear memory within the conditioning paradigm developed by Spring et al. (2015). The conditioned fear response was measured by changes in SC. We hypothesized that oxytocin would reduce the SC response to a previously conditioned stimulus when this stimulus is reactivated (CS+R) in the presence of the drug, compared to a previously conditioned stimulus that is not reactivated (CS+N).

Method

Participants

Prior to enrollment, participants were screened by phone to verify the presence of a manageable, non-phobic fear of spiders as determined by scores above the mean on the SPQ-15 (Olatunji et al., 2009) and absence of phobia criteria extracted from the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-IV; First, Spitzer, Gibbon, & Williams, 1997). Participants also underwent a set of screening criteria taken directly from the SCID-IV to verify absence of current psychiatric disorders, serious medical or neurological conditions, brain injury, and current or past substance abuse. A positive response to a screening criterion led to a full examination of that criterion per SCID-IV. A urine drug screen verified the absence of illicit substances and psychotropic medications. The presence of a current Axis I psychiatric disorder or illicit substances/medications was grounds for withdrawal from the study.

Seven healthy participants (6 females, 1 male) were enrolled in the study. Of these, none had an unmeasurable (very low) SC level or a current Axis I psychiatric disorder. Following the Day 1 procedure, 1 subject was withdrawn due to failure to demonstrate adequate differential conditioning (conditioning criteria are described below in Data Reduction). One additional subject was withdrawn due to improper self-administration of the study medication. The remaining 5 participants (all females) who underwent study procedures on Days 1-3 had a mean age of 22.80 years (SD = 3.27, range 18 to 27 years) and a mean score on the SPQ-15 of 7.00 (SD = 2.55, range 5 to 11 of a possible 0 to 15). Mean years of education was 16.20 (SD = 1.79, range 14 to 19 years).

The study protocol was approved by the Partners Human Research Committee (PHRC), as well as the United States Army Medical Research and Material Command (USAMRMC) Human Research Protection Office (HRPO). After a full explanation of the procedures, all participants provided written informed consent.

Equipment and Stimuli

Equipment and stimuli were the same as those used by Spring and colleagues (Spring et al., 2015). Skin conductance analog signals were recorded using a Coulbourn Lab Linc V Series Human Measurement System (Coulbourn Instruments, Whitehall, PA) with a Coulbourn Isolated Skin Conductance Coupler (V71-23) through 8mm (sensor diameter) Ag/AgCl electrodes (In Vivo Metric; Healdsburg, CA) filled with an isotonic paste. Electrodes were separated by 14mm, as determined by the width of the adhesive collar, and placed on the hypothenar surface of the subject's non-dominant hand in accordance with published guidelines (Boucsein et al., 2012; Fowles et al., 1981). The SC signal was sampled at 1000 Hz and digitized by a Coulbourn Analog to Digital Converter (V19-16). A Cobalt notebook computer (IBM-compatible; Cobalt Computers, Whitehall, PA) with custom-designed software was used to record and store the digitized physiological signals.

Nine high-definition video clips (Virtually Better Inc., Decatur, GA) depicting one of three tarantulas occupying one of three contexts comprised the conditioned stimuli (CS). Two of the three tarantulas always served as the CS+s, either the to-be-reactivated CS+ (CS+R) or the to-be-non-reactivated CS+ (CS+N), and were paired with the unconditioned stimulus (US, shock) on day 1. The to-be CS+R served as the stimulus that was presented on day 2 after receiving the study medication; the to-be CS+N was not presented on day 2. The third tarantula served as the

CS- and was not paired with the US and not presented on day 2. The three contexts within which the tarantulas appeared were a kitchen (A), bedroom (B) and office (C). The particular tarantula that served as the CS+N or CS+R was counterbalanced across participants; the tarantula used to represent the CS- was the same across subjects.

The US was a 0.5-sec mild electric shock ranged in intensity (0.2 to 4.0 milliamperes) according to the level determined by the participant to be "highly annoying but not painful." The US was delivered using a Coulbourn Transcutaneous Aversive Finger Stimulator (E13-22) through shock electrodes attached to the middle segments of the 2nd and 3rd fingers on the hand opposite to that on which the SC recording electrodes were attached.

Video clips lasted 12 seconds: four seconds of context alone (i.e., no tarantula), followed by eight seconds of context plus tarantula. On reinforced trials, the US immediately followed the CS+. The intertrial interval consisted of a black screen and was randomized to last 16, 18, 20, 22, or 24 seconds. The procedure was implemented using E-Prime Professional 2.0 (Psychology Software Tools, Inc., Sharpsburg, PA).

Procedure

As depicted in Figure 10, the procedure consisted of a differential fear-conditioning paradigm that entailed laboratory visits over three consecutive days. On day 1, participants were instructed: *"Today, you will be viewing videos of spiders on the television, and you will receive electric shocks on your fingers after viewing some of the spiders. These shocks will be annoying, but not painful. We will also use electrodes on your palm to record how your body responds to this procedure."* Following these instructions, participants set the shock to a level to be "highly annoying but not painful" (Orr, Metzger, Lasko, Macklin, Peri & Pitman, 2000). Participants were then shown still images, i.e., screenshots, of the three tarantulas that would serve as CSs, accompanied by these instructions: *"During the experiment, it will be important that you are able to tell these spiders apart. To do this, try focusing on the legs. For this spider, note the alternating black and white stripe pattern. For this spider, note the orange highlights. For this spider, note that the legs are solid black."* Prior to beginning the procedure, the lights were dimmed and over-ear headphones placed on the participant to reduce ambient noise and enable communication with study staff in the next room. Participants were instructed to sit still in the chair, keep their eyes open, and be attentive to the stimuli presented on the screen. Next, there was a 5-min baseline period to record physiological levels.

Day 1 consisted of two sequential phases: 1) unreinforced presentations of each of the nine possible spider-context combinations in pseudorandom order (*habituation*), and 2) eight partially reinforced (i.e., five of eight) presentations each of CS+R and CS+N, presented separately in blocks and interspersed pseudorandomly with eight presentations of CS- (*acquisition*). Presentation order of CS+R and CS+N trial blocks was counterbalanced across participants. All CS+R, CS+N, and CS- presentations during acquisition occurred within context A. Participants who did not meet the defined cutoff for demonstrating a differential conditioned response (see below) were withdrawn prior to day 2.

The procedures for days 2, 3, and the 1-month follow-up were largely the same as for day 1, with the following exceptions: a) the procedure for setting the level of shock was not repeated, as the shock level determined on the first visit was used for the remainder of the study, b)

participants were only familiarized with images of the stimuli prior to undergoing the day 1 procedure, and c) rather than “will receive” as on day 1, participants were instructed that they “may or may not receive” electric shocks.

Day 2 consisted of the participant receiving a 32 IU dose of intranasal oxytocin (Syntocinon Nasal Spray, Novartis, Basel, Switzerland), which was followed 30 minutes later by a single, unreinforced presentation of the CS+R (*reactivation*). Reactivation of the CS+R occurred in context B. Oxytocin is a peptide that is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus and projected to the posterior pituitary and limbic areas that include the hippocampus, amygdala, striatum, hypothalamus, locus coeruleus and other areas (Sofroniew, 1983). The participant self-administered the oxytocin intranasally.

Day 3 consisted of three sequential phases: 1) two unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*renewal test*); and 2) three unsignalled presentations of the US alone, followed by 3) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*reinstatement test trials* and *extinction*). All presentations of the CSs occurred in context A. Ordering of CS+R and CS+N presentations, within the full set of trials that included CS- presentations, was counterbalanced across subjects.

The one-month follow-up consisted of two phases: 1) ten unreinforced presentations each of the CS+R, CS+N, and CS- pseudorandomly interspersed (*spontaneous recovery test* and *re-extinction*), followed by 2) eight partially-reinforced, i.e., five of eight, presentations each of CS+R and CS+N presented in successive blocks and interspersed with eight CS- trials for the respective blocks as was done on day 1 (*re-acquisition/savings test*). All stimuli were presented in context C during this visit, and the CS+R block of trials was presented first.

Physiological Measures and Data Reduction

As previously described (Milad, Orr, Pitman, & Rauch, 2005; Orr et al., 2000), an SCR for the CS interval was calculated for each trial by subtracting the mean SC level during the two sec prior to CS onset (context alone presentation) from the peak SC level during the eight sec CS interval. These SCR values reflect change in skin conductance level beyond that resulting from presentation of context alone. A square root transformation was applied to the absolute value of each SCR, followed by replacement of the + or - sign, prior to statistical analysis.

For day 1, the untransformed SCR data were scored to determine whether a definable differential SCR was obtained for *both* the CS+R and CS+N during the acquisition phase. We averaged SCRs across respective CS+R, CS+N, and CS- trials in order to calculate a difference score between the CS+R and its respective CS- trials and between the CS+N and its respective CS- trials. A cutoff of .1μS was applied to each difference score and participants with one or both difference scores below this cutoff were withdrawn from the study prior to Day 2.

Results

The primary statistical approach was mixed-model, repeated measures analysis of variance (ANOVA), performed separately for each experimental phase. Participants were treated as a random effect, Stimulus (CS+R, CS+N, CS-) as a within-participants effect, and Trials as the repeated measure.

Acquisition Phase (day 1).

Responses to the CSs during the acquisition phase were examined across the 8 presentations of each CS using a mixed-model repeated measures ANOVA with two factors: Stimulus (CS+R, CS- or CS+N, CS-) and Trials. As can be seen in Table 4 and Figure 11, top panel, during the acquisition phase, the CS+R and CS+N demonstrated larger SCRs, compared to their respective CS- trials. There was no difference in SCR magnitudes to CS+R and CS+N trials. There was a significant Stimulus x Trials interaction for comparison of CS+R to CS- ($F(7, 28) = 2.60$, $p = .034$, $Eta^2 = .10$), but not for comparisons of CS+N to CS- ($F(7, 28) < 1$, p = NS, $Eta^2 = .02$) and CS+R to CS+N ($F(7, 28) = 1.67$, $p = .16$, $Eta^2 = .14$).

SC responses for the US interval, which represented the unconditioned response (UR), were calculated and plotted for the acquisition phase (Figure 12). Because SCR onset has a known latency of 1-2 s (Edelberg, 1967), the 1-s interval immediately following US onset was used as the baseline for calculating the SC UR, which was subtracted from the peak SC level within the 6-s interval following US onset to yield the UR. A square root transformation was applied to the UR, as was done for the CR. The statistical model was an ANOVA with Stimulus (CS+R, CS+N, CS-) as a within-participants effect and Trials as the repeated measure. Mixed-model ANOVA could not be used for these comparisons due to too many likelihood evaluations for one of the comparisons. Consequently, fixed-effects ANOVAs were used for the comparisons. As expected, both CS+R and CS+N trials produced larger SCRs during the US interval, compared to CS- trials ($F(1, 64) = 118.98$, $p < .001$, $Eta^2 = .45$; $F(1, 64) = 233.47$, $p < .001$, $Eta^2 = .51$; respectively). There were significant Stimulus x Trials interactions for both CS+R and CS+N trials ($F(7, 64) = 6.68$, $p < .001$, $Eta^2 = .18$; $F(7, 64) = 12.96$, $p < .001$, $Eta^2 = .20$; respectively). The average UR magnitudes for CS+R and CS+N reinforced trials did not differ ($F(1, 64) < 1$, p = NS). There was a significant Stimulus x Trials interaction for the comparisons of CS+R to CS+N trials ($F(7, 64) = 3.64$, $p = .002$, $Eta^2 = .11$, Figure 12).

Renewal Phase (Day 3).

Responses to the CSs during the renewal phase were examined over the 2 presentations of each CS using a two-factor (Stimulus, Trials) repeated measures ANOVA. The Stimulus factor has two levels in each of three ANOVAs that compared CS+R to CS- trials, CS+N to CS- trials and CS+R to CS+N trials; the Trials factor has 2 levels. As can be seen in Table 1and Figure 11, bottom panel, SCR magnitudes to CS+R and CS+N presentations did not significantly differ from those to the CS- and did not differ from each other. This suggests that the conditioned fear response to the CS+R and CS+N did not persist beyond the Day2 intervention even though only the CS+R was presented following administration of oxytocin. The Stimulus x Trials interaction effects were not significant for the comparison of CS+R to CS- trials ($F(1, 4) < 1$, p = NS, $Eta^2 = .00$) and of CS+N to CS- trials ($F(1, 4) < 1$, p = NS, $Eta^2 = .01$).

Reinstatement Phase (Day 3).

Responses to the CSs during the reinstatement and extinction phase, which immediately followed the unsignalled shock presentations, were first examined across the 10 presentations of each CS using a two-factor (Stimulus, Trials), repeated measures model. The Stimulus factor has three levels (CS+R, CS+N, CS-); the Trials factor has 10 levels. The main effect of Stimulus ($F(2, 8) < 1$, p = NS) and the Stimulus x Trials interaction ($F(18, 72) < 1$, p = NS) were not significant. For the interested reader, direct comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials are provided in Table 4. As can be seen in Table 4 and Figure 11, bottom

panel, the comparisons of CS+R to CS- and CS+N to CS- SCR magnitudes did not yield significant differences. The Stimulus x Trials interaction effects were also not significant for the comparisons of CS+R to CS- trials ($F(9, 36) < 1$, p = NS), CS+N to CS- trials ($F(9, 36) < 1$, p = NS) and CS+R to CS+N trials ($F(9, 36) < 1$, p = NS).

In order to avoid the potential dampening of differences in SCR to the CSs due to rapid extinction, we also performed two-factor (Stimulus, Trials), mixed-model repeated measures ANOVA that only considered the first two presentations of each CS during the reinstatement phase. For these analyses, the Stimulus factor has 2 levels in the comparisons of CS+R to CS-, CS+N to CS-, and CS+R to CS+N trials and the Trials factor has 2 levels. As can be seen in Table 1, SCR magnitudes to initial presentations of the CS+R and CS+N were not significantly larger than those to the CS- and there was no difference when directly comparing CS+R and CS+N trials.

Discussion

Brief clips of moving tarantulas depicted within various contexts served as the conditioned stimuli for fear-conditioned SCRs. As previously observed by Spring et al. (2015), the differential conditioning procedure, combined with setting a differential conditioning threshold for SCRs, produced robust responses to the conditioned stimuli that would subsequently serve as the reactivated (CS+R) and non-reactivated (CS+N) cues, by which reconsolidation blockade of conditioned fear responses was assessed. On the day following fear conditioning, reconsolidation blockade of the CR was attempted by administering oxytocin followed 30 min later by a single reactivation presentation of one of the two CS+s (CS+R). On subsequent testing the next day (Renewal Phase), we found that neither, the CS+R nor the non-reactivated CS+ (CS+N), produced significantly larger SCRs than to the CS- and that the responses to the CS+R and CS+N did not differ from each other. Furthermore, following a series of unsignalled shocks (Reinstatement phase), the difference between the magnitude of SCRs to the CS+R and CS- and between the CS+N and CS- remained non-significant. These results suggest that oxytocin reduced reconsolidation of the fear response to the CS+R, that oxytocin's effect may have generalized to the non-reactivated, CS+N and that the effect persisted when attempting to reinstate the conditioned fear response.

The very small sample size of the present study provides limited statistical power. However, the Renewal phase effect sizes associated with the comparisons of the CS+R with the CS- and the CS+N with the CS- were both small ($Eta^2 = .05$) and roughly 1/3 to 1/4 the effect sizes reported by Spring et al. (2015) and the two studies above (Frichionne et al., included in report; Orr et al., included in report). The exception to the larger effect sizes observed in the other studies is the comparison of CS+R with CS- in the Renewal phase of the Orr et al. study, in which mifepristone appeared to reduce the conditioned fear response to the CS+R. The effect size for this latter comparison was $Eta^2 = .08$, which is still somewhat larger than the effect sizes observed in the present study. It seems unlikely that reduced responding to the CS+R and CS+N during the Renewal phase on Day 3 is due to weaker conditioning in the present study. The effect sizes associated with comparisons between the CS+R and CS- ($Eta^2 = .22$) and between the CS+N and CS- ($Eta^2 = .22$) during fear conditioning on Day 1of the present study are within the range of effect sizes for fear acquisition observed by Spring et al. (2015) and the two studies above (Frichionne et al., included in report; Orr et al., included in report) using the same conditioning procedure.

The present findings for oxytocin contrast with the negative findings for propranolol (Spring et al., 2015) and a behavioral intervention (Frichionne et al., in this report), which failed to demonstrate any reduction of a conditioned fear response using the same conditioning procedure as used in the present study. As reported above, when a conditioned stimulus for fear was reactiviated after receiving mifepristone, fear was more greatly reduced to the reactivated CS+ than to a non-reactivated CS+ (Orr et al., in this report). This suggests that mifepristone has a selective effect on fear reduction, i.e., it requires that the particular fear memory be reactivated in order for there to be a reduction by mifepristone. In contrast, the present findings suggest that oxytocin may have a non-selective effect on fear reduction, such that fear reduction that occurs to a reactivated fear stimulus will generalize to a fear stimulus that is not reactivated. Because of the very small sample size, the finding for oxytocin in the present study must be viewed with considerable caution. However, if the finding proves reliable, it would suggest that oxytocin could be particularly useful in reducing clinical fears associated with exposure to multiple and varied traumatic events, such as might occur in combat veterans.

Table 4: Results of mixed-model repeated measures ANOVA of SCR ($\sqrt{\mu S}$) for the CS interval.

			DF	F	p	Eta^2
Day 1	Acquisition	CS+R vs CS-	1, 4	11.13	.029	0.22
		CS+N vs CS-		16.94	.015	0.22
		CS+R vs CS+N		<1	NS	0.01
Day 3	Renewal	CS+R vs CS-	1, 4	<1	NS	0.05
		CS+N vs CS-		<1	NS	0.05
		CS+R vs CS+N		<1	NS	0.00
	Reinstatement (2 trials)	CS+R vs CS-	1, 4	<1	NS	0.01
		CS+N vs CS-		<1	NS	0.02
		CS+R vs CS+N		<1	NS	0.00
	Reinstatement and Extinction (10 trials)	CS+R vs CS-	1, 4	<1	NS	0.02
		CS+N vs CS-		<1	NS	0.02
		CS+R vs CS+N		<1	NS	0.01

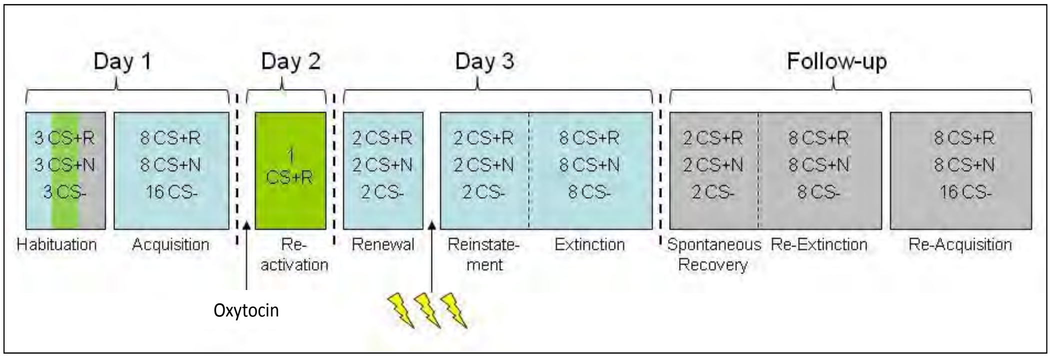

Figure 10: Depiction of the 4-session fear-conditioning procedure. Fear conditioning occurred on day 1. A single dose of 32 IU of intranasal oxytocin was administered on day 2, followed by reactivation of one of the two the CS+s (i.e., the CS+R). On day 3 and the 1-month follow-up visit, post-intervention reactivity to the conditioned stimuli was tested. CS+R = CS+ to-be-reactivated; CS+N = CS+ non-reactivated; CS- = unreinforced. Lightning bolts represent unsignalled presentations of the US alone. Shading colors represent the context in which the stimuli were presented: blue = A, green = B, grey = C. CS+R and CS+N acquisition and re-acquisition trials occurred in blocks as described in the text.

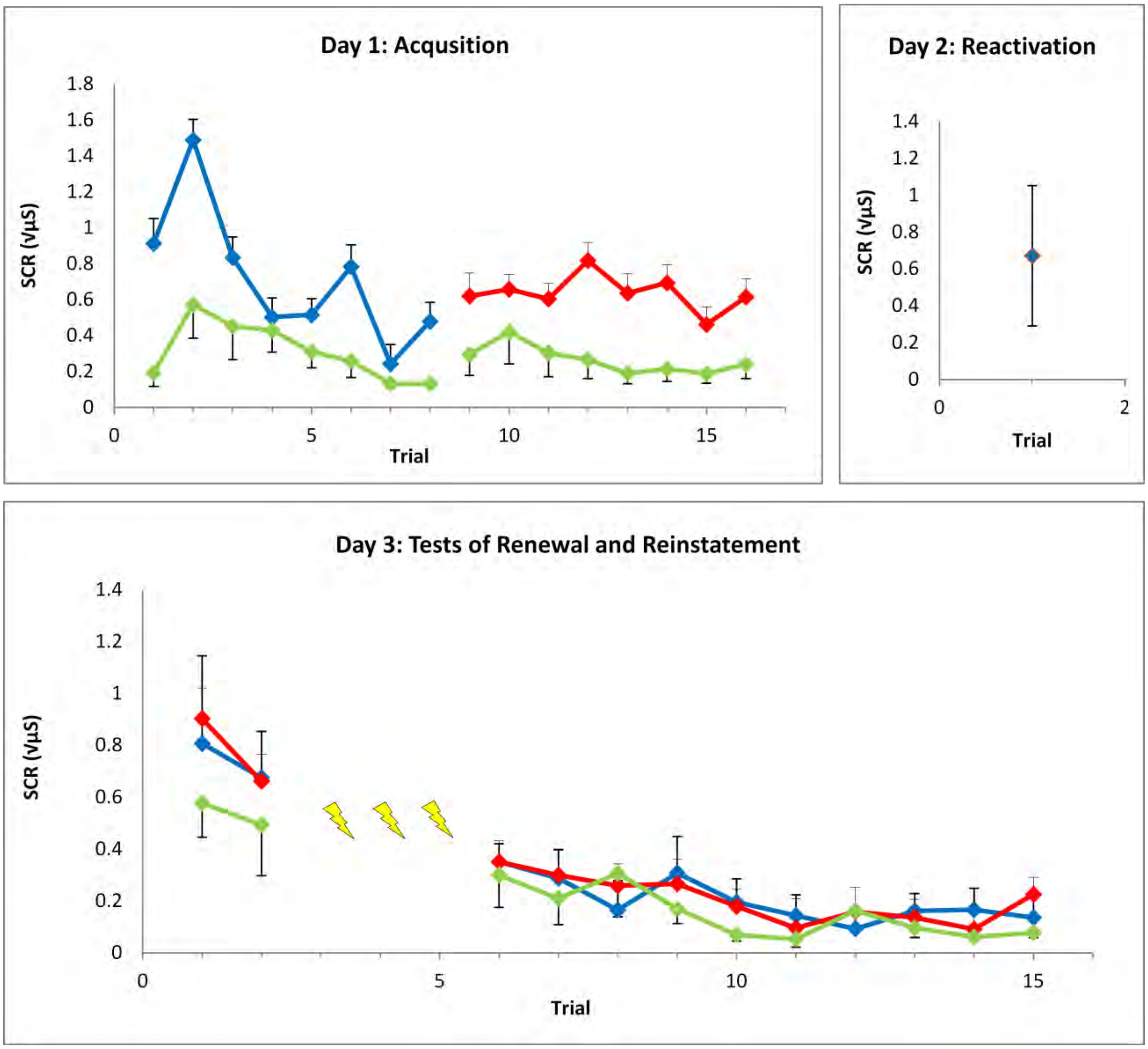

Figure 11. Group mean skin conductance responses to CS+R, CS+N, and CS- trials for the Acquisition (day 1), Reactivation (day 2), and Renewal and Reinstatement (day 3) phases. Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Lightning bolts represent unsignaled presentations of the US alone. SCR = skin conductance response.

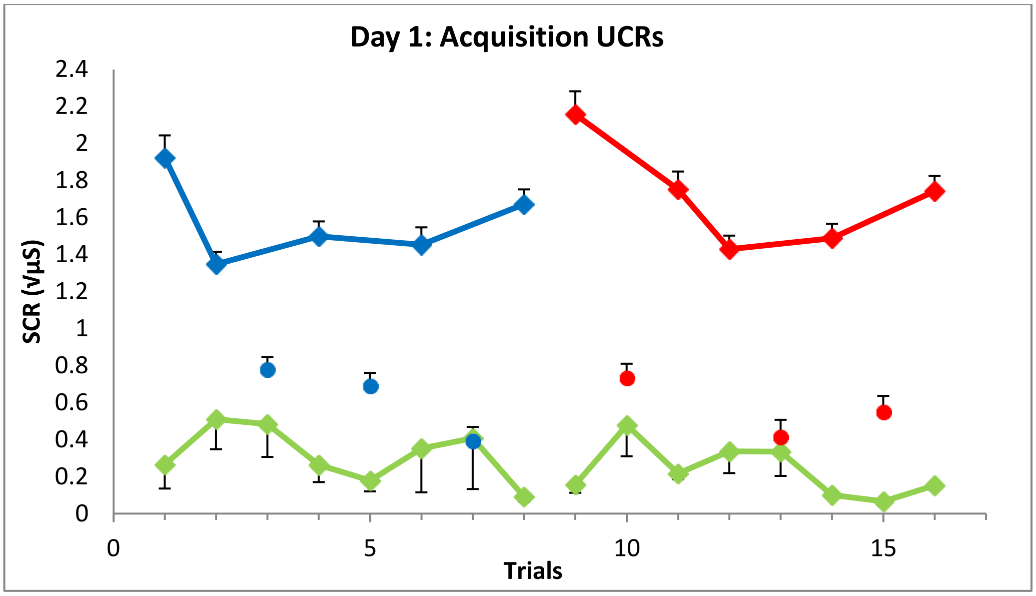

Figure 12. Group mean skin conductance responses during US interval following CS+R, CS+N, and CS- trials for the Acquisition phase (day 1). Bars represent standard errors. Color represents stimulus: blue = CS+R, red = CS+N, green = CS-. Unconnected circles represent unreinforced (i.e., no shock) CS+ trials. SCR = skin conductance response.

3. KEY RESEARCH ACCOMPLISHMENTS

-Developed an experimental assay that produced optimal Pavlovian differential fear conditioning for testing the relative strengths of various novel behavioral and pharmacological reconsolidation-based interventions. This conditioning protocol produces robust acquisition of skin conductance responses to two different fear cues that allow for within-subjects testing of the efficacy of reconsolidation-based interventions.

-Tested propranolol as a reconsolidation-based pharmacological intervention.

-Tested delayed extinction as a reconsolidation-based behavioral intervention.

-Tested mifepristone as a reconsolidation-based pharmacological intervention.

-Preliminary testing of oxytocin as a reconsolidation-based pharmacological intervention.

-Publication: Spring J, Wood N., Mueller-Pfeiffer C, Milad MR, Pitman RK, Orr SP. Pre-reactivation propranolol fails to reduce skin conductance reactivity to fear-conditioned stimuli. *Psychophysiology*, 2015;52(3):407-415. doi: 10.1111/psyp.12326

- Poster: presented at the Society of Biological Psychiatry meeting in May 2013

4. REPORTABLE OUTCOMES

Results from the propranolol intervention were found to be negative and have recently been published in the journal *Psychophysiology* (Spring, Wood, Mueller-Pfeiffer, Milad, Pitman & Orr, 2015). These findings were also reported in May 2013 at the annual Society of Biological Psychiatry Meeting in San Francisco, California.

Results from the delayed-extinction behavioral intervention were also negative and are provided above in this Final Report. A manuscript is in preparation for publication of these negative findings.

Results from the mifepristone intervention are encouraging and suggest that this drug may reduce reconsolidation of a conditioned fear memory. However, differences in the strength of initial conditioning to the two fear cues (CS+R, CS+N) required that an adjustment be made when testing for the reconsolidation effect. Thus, the findings ought to be viewed with caution. Subject recruitment for this project was especially challenging, as many potential participants did not want to take mifepristone due to its potential side effects.

Results from the oxytocin intervention raise the possibility that this drug reduces conditioned fear to a non-reactivated fear cue (CS+N), as well as to the reactivated fear cue (CS+R), i.e., the effect was generalized. This study was initiated towards the end of the funding period and there were delays in getting access to the oxytocin due to its being shipped from Switzerland. Consequently,

only a few subjects could be enrolled and tested before funding for the project ended. The results should be viewed as very tentative, albeit interesting.

5. CONCLUSION

The data and findings obtained to date suggest that our goal of creating a modified fear-conditioning paradigm that is free from intervention floor effects associated with blockade of memory reconsolidation was achieved. While the paradigm has been successful, results obtained on Day 3 in the propranolol and behavioral intervention groups suggest that 40mg of propranolol and delayed extinction do not block reconsolidation of the fear memory. However, results from the mifepristone and oxytocin interventions hold some promise in being able to reduce fear memory reconsolidation.

6. REFERENCES

Agren, T., Engman, J., Frick, A., Björkstrand, J., Larsson, E.-M., Furmark, T., & Fredrikson, M. (2012). Disruption of reconsolidation erases a fear memory trace in the human amygdala. *Science (New York, N.Y.)*, *337*(6101), 1550–2. doi:10.1126/science.1223006

Boucsein, W. (2012). *Electrodermal Activity*. Boston, MA: Springer US. doi:10.1007/978-1-4614-1126-0

Boucsein, W., Fowles, D. C., Grimnes, S., Ben-Shakhar, G., Roth, W. T., Dawson, M. E., & Filion, D. L. (2012). Publication recommendations for electrodermal measurements. *Psychophysiology*, *49*, 1017–34. doi:10.1111/j.1469-8986.2012.01384.x

Bradley, M. M., Cuthbert, B. N., & Lang, P. J. (1990). Startle reflex modification: emotion or attention? *Psychophysiology*, *27*(5), 513–22. PMID: 2274614

Brunet, A., Orr, S. P., Tremblay, J., Robertson, K., Nader, K., & Pitman, R. K. (2008). Effect of post-retrieval propranolol on psychophysiologic responding during subsequent script-driven traumatic imagery in post-traumatic stress disorder. *Journal of Psychiatric Research*, *42*(6), 503–6. doi:10.1016/j.jpsychires.2007.05.006

Brunet, A., Thomas, E., Saumier, D., Ashbaugh, A., Azzoug, A., Pitman, R., ...Tremblay, J. (2014). Trauma reactivation plus propranolol is associated with durably low physiological responding during subsequent script-driven imagery. *Canadian Journal of Psychiatry*, 59, in press.

Debiec, J., & Ledoux, J. E. (2004). Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience*, *129*(2), 267–72. doi:10.1016/j.neuroscience.2004.08.018

Di Simplicio, M., Massey-Chase, R., Cowen, P. J. & Harmer, C. J. (2009). Oxytocin enhances processing of positive versus negative emotional information in healthy male volunteers. *Journal of Psychopharmacology, 23*(3), 241-248. doi:10.1177/0269881108095705

Dudai, Y. (2004). The neurobiology of consolidations, or, how stable is the engram? *Annual Review of Psychology*, *55*, 51–86. doi:10.1146/annurev.psych.55.090902.142050

Duvarci S. & Nader K. (2004). Characterization of fear memory reconsolidation. *Journal of Neuroscience, 24*, 9269–9275. Doi:10.1523/jneurosci.2971-04.2004

Edelberg, R. (1967). Electrical properties of the skin. In C. C. Brown (Ed.), *Methods in psychophysiology* (pp. 1–53). Baltimore: Williams & Wilkins.

Esteves, F., Parra, C., Dimberg, U., & Ohman, A. (1994). Nonconscious associative learning: Pavlovian conditioning of skin conductance responses to masked fear-relevant facial stimuli. *Psychophysiology*, *31*(4), 375–85. PMID: 10690918

First, M. B., Spitzer, R. L., Gibbon, M., & Williams, J. B. W. (1997). *User's Guide for the Structured Clinical Interview for Dsm-IV Axis I Disorders: Scid-1 Clinician Version* (p. 132). Amer Psychiatric Pub. Retrieved from http://www.amazon.com/Structured-Clinical-Interview-Dsm-IV-Disorders/dp/0880489316

Flykt, A., Esteves, F., & Ohman, A. (2007). Skin conductance responses to masked conditioned stimuli: phylogenetic/ontogenetic factors versus direction of threat? *Biological Psychology*, *74*(3), 328–36. doi:10.1016/j.biopsycho.2006.08.004

Fowles, D. C., Christie, M. J., Edelberg, R., Grings, W. W., Lykken, D. T., & Venables, P. H. (1981). Committee report. Publication recommendations for electrodermal measurements. *Psychophysiology*, *18*, 232–239. doi:10.1111/j.1469-8986.2012.01384.x

Gilman, A., & Goodman, L. (1996). *Goodman and Gilman's: The pharmacological basis of therapeutics*. New York: McGraw-Hill.

Golkar, A., Bellander, M., Olsson, A. & Ohman, A. (2012). Are fear memories erasable?-reconsolidation of learned fear with fear-relevant and fear irrelevant stimuli. *Frontiers in Behavioral Neuroscience, 6*(80), 1-10. doi:10.3389/fnbeh.2012.00080

Graham, S., J., Scaife, J. C., Langley, R. W., Bradshaw, C. M., Szabadi, E., & Xi, L. (2005). Effects of lorazepam on fear-potentiated startle responses in man. *Journal of Psychopharmacology,* ***19,*** 249–258. PMID: 15888510

Hamm, A. O., Greenwald, M. K., Bradley, M. M., Cuthbert, B. N., & Lang, P. J. (1991). The fear potentiated startle effect. Blink reflex modulation as a result of classical aversive conditioning. *Integrative Physiological and Behavioral Science : The Official Journal of the Pavlovian Society*, *26*(2), 119–26. PMID: 1878318

Heinrichs, M., Meinlschmidt, G., Wippich, W., Ehlert, U. & Hellhammer, D. H. (2004). Selective amnesic effects of oxytocin on human memory. *Physiology and Behavior, 83*(1), 31-38. doi:10.1016/j.physbeh.2004.07.020

Hoge, E. A, Worthington, J. J., Nagurney, J. T., Chang, Y., Kay, E. B., Feterowski, C. M., ...Pitman, R. K. (2012). Effect of acute posttrauma propranolol on PTSD outcome and physiological responses during script-driven imagery. *CNS Neuroscience & Therapeutics*, *18*, 21–7. doi:10.1111/j.1755-5949.2010.00227.x

Holmes, E. A., James, E. L., Coode-Bate, T., & Deeprose, C. (2009). Can playing the computer game “Tetris” reduce the build-up of flashbacks for trauma? A proposal from cognitive science. *PloS One*, *4*(1), e4153. doi:10.1371/journal.pone.0004153

Huber, D., Veinante, P. & Stoop, R. (2005). Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. *Science, 308*(5719), 245-248. doi:10.1126/science.1105636

Jin, X.-C., Lu, Y.-F., Yang, X.-F., Ma, L., & Li, B.-M. (2007). Glucocorticoid receptors in the basolateral nucleus of amygdala are required for postreactivation reconsolidation of auditory fear memory. *The European Journal of Neuroscience*, *25*(12), 3702–12. doi:10.1111/j.1460-9568.2007.05621.x

Kindt, M., & Soeter, M. (2013). Reconsolidation in a human fear conditioning study: a test of extinction as updating mechanism. *Biological Psychology*, *92*(1), 43–50. doi:10.1016/j.biopsycho.2011.09.016

Kindt, M., Soeter, M., & Vervliet, B. (2009). Beyond extinction: erasing human fear responses and preventing the return of fear. *Nature Neuroscience*, *12*(3), 256–8. doi:10.1038/nn.2271

Krauseneck, T., Padberg, F., Roozendaal, B., Grathwohl, M., Weis, F., Hauer, D., ...Schelling, G. (2010). A beta-adrenergic antagonist reduces traumatic memories and PTSD symptoms in female but not in male patients after cardiac surgery. *Psychological Medicine*, *40*, 861–869. doi:10.1017/S0033291709990614

Lee, J. L. C., Everitt, B. J., & Thomas, K. L. (2004). Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science (New York, N.Y.)*, *304*(5672), 839–43. doi:10.1126/science.1095760

McGaugh, J. L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience*, *27*, 1–28. doi:10.1146/annurev.neuro.27.070203.144157

Milad, M. R., Orr, S. P., Pitman, R. K., & Rauch, S. L. (2005). Context modulation of memory for fear extinction in humans. *Psychophysiology*, *42*(4), 456–64. doi:10.1111/j.1469-8986.2005.00302.x

Mineka, S., & Öhman, A. (2002). Phobias and preparedness: the selective, automatic, and encapsulated nature of fear. *Biological Psychiatry*, *52*(10), 927–937. doi:10.1016/S0006-3223(02)01669-4.

Monfils, M-H., Cowansage, K. K., Klann, E. & Ledoux, J. E. (2009). Extinction-reconsolidation boundaries : Key to persistent attenuation of fear memories. *Science, 324*(5929), 951-955. doi: 10.1126/science.1167975

Nader, K., & Einarsson, E. O. (2010). Memory reconsolidation: an update. *Annals of the New York Academy of Sciences*, *1191*, 27–41. doi:10.1111/j.1749-6632.2010.05443.x

Nader, K., Schafe, G. E., & Le Doux, J. E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, *406*(6797), 722–6. doi:10.1038/35021052

Nugent, N. R., Christopher, N. C., Crow, J. P., Browne, L., Ostrowski, S., & Delahanty, D. L. (2010). The efficacy of early propranolol administration at reducing PTSD symptoms in pediatric injury patients: a pilot study. *Journal of Traumatic Stress*, *23*(2), 282–7. doi:10.1002/jts.20517

Olatunji, B. O., Woods, C. M., de Jong, P. J., Teachman, B. A., Sawchuk, C. N., & David, B. (2009). Development and initial validation of an abbreviated Spider Phobia Questionnaire using item response theory. *Behavior Therapy*, *40*(2), 114–30. doi:10.1016/j.beth.2008.04.002

Orr, S. P., Metzger, L. J., Lasko, N. B., Macklin, M. L., Peri, T., & Pitman, R. K. (2000). De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *Journal of Abnormal Psychology*, *109*(2), 290–8. PMID: 10895567

Oyarzun, J. P., Lopez-Barroso, D., Fuentemilla, L., Cucurell, D., Pedraza, C., Rodriguez-Fornells, A. et al. (2012). Updating fearful memories with extinction training during reconsolidation: a human study using auditory aversive stimuli. *PLoS ONE* 7:e38849. Doi:10.1371/journal.pone.0038849

Pitman, R. K. (1989). Post-traumatic stress disorder, hormones, and memory. *Biological Psychiatry*. doi:10.1016/0006-3223(89)90033-4

Pitman, R. K., Milad, M. R., Igoe, S. A., Vangel, M. G., Orr, S. P., Tsareva, A., ...Nader, K. (2011). Systemic mifepristone blocks reconsolidation of cue-conditioned fear; propranolol prevents this effect. *Behavioral Neuroscience*, *125*(4), 632–8. doi:10.1037/a0024364

Pitman, R. K., Sanders, K. M., Zusman, R. M., Healy, A. R., Cheema, F., Lasko, N. B., ...Orr, S. P. (2002). Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. *Biological Psychiatry*, *51*(2), 189–92. PMID: 11822998

Przybyslawski, J., Roullet, P., & Sara, S. J. (1999). Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(15), 6623–8. PMID: 10414990

SAS Institute Inc. (2012). *SAS® 9.3*. Cary, NC: SAS Institute Inc.

Schiller, D., Monfils, M.-H., Raio, C. M., Johnson, D. C., Ledoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature*, *463*(7277), 49–53. doi:10.1038/nature08637

Sevenster, D., Beckers, T., Kindt, M., 2012. Retrieval per se is not sufficient to trigger reconsolidation of human fear memory. Neurobiology of Learning and Memory 97, 338-345.

Soeter, M., & Kindt, M. (2010). Dissociating response systems: erasing fear from memory. *Neurobiology of Learning and Memory*, *94*(1), 30–41. doi:10.1016/j.nlm.2010.03.004

Soeter, M., & Kindt, M. (2011). Disrupting reconsolidation: pharmacological and behavioral manipulations. *Learning & Memory, 18*(6), 357–66. doi:10.1101/lm.2148511

Soeter, M., Kindt, M., 2013. High trait anxiety: a challenge for disrupting fear memory reconsolidation. PLOS One. 8, e75239.

Sofroniew, M. V. (1983). Morphology of vasopressin and oxytocin neurons and their central and vascular projections. *Progress in Brain Research, 60*, 101-114.

Spring, J., Wood, N., Mueller-Pfeiffer, C., Milad, M. R., Pitman, R. K., & Orr S. P. (2015). Pre-reactivation propranolol fails to reduce skin conductance reactivity to fear-conditioned stimuli. *Psychophysiology*, *52*(3):407-415. doi:10.1111/psyp.12326

Stein, M. B., Kerridge, C., Dimsdale, J. E., & Hoyt, D. B. (2007). Pharmacotherapy to prevent PTSD: Results from a randomized controlled proof-of-concept trial in physically injured patients. *Journal of Traumatic Stress*, *20*(6), 923–32. doi:10.1002/jts.20270

Suzuki, A., Josselyn, S. A., Frankland, P. W., Massushige, S., Silva, A. J., & Kida, S. (2004). Memory reconsolidation and extinction have distinct temporal and biochemical signatures. Journal of Neuroscience, 24, 4787-4795. doi: 10.1523/jneurosci.5491-03.2004

Taubenfeld, S.M., Riceberg, J.S., New, A.S., Alberini, C.M., 2009. Preclinical assessment for selectively disrupting a traumatic memory via postretrieval inhibition of glucocorticoid

Vaiva, G., Ducrocq, F., Jezequel, K., Averland, B., Lestavel, P., Brunet, A., & Marmar, C. R. (2003). Immediate treatment with propranolol decreases posttraumatic stress disorder two months after trauma. *Biological Psychiatry*, *54*(9), 947–9. PMID: 14573324

Walker, M. P., Brakefield, T., Hobson, J. A., & Stickgold, R. (2003). Dissociable stages of human memory consolidation and reconsolidation. *Nature, 425*, 616–620. doi:10.1038/nature01930

Wallin, B. G. (1981). Sympathetic nerve activity underlying electrodermal and cardiovascular reactions in man. *Psychophysiology*, *18*(4), 470–6. PMID: 7267931

Wang, S. H., Alvares, L. D., & Nader, K. (2009). Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation. *Nature Neuroscience, 12*, 905-912. doi:10.1038/nn.2350

Williams, L. M., Phillips, M. L., Brammer, M. J., Skerrett, D., Lagopoulos, J., Rennie, C., … Gordon, E. (2001). Arousal dissociates amygdala and hippocampal fear responses: evidence from simultaneous fMRI and skin conductance recording. *NeuroImage*, *14*, 1070–1079. doi:10.1006/nimg.2001.0904

Wood, N. E., Rosasco, M. L., Suris, A. M., Spring, J. D., Marin, M. F., Lasko, N. B., Goetz, J. M., Fischer, A. M., Orr, S. P., Pitman, R. K. (2015). Pharmacological blockade of memory reconsolidation in posttraumatic stress disorder: three negative psychophysiological studies. *Psychiatry Research, 225*(1-2), 31-39. doi:10.1016/j.psychres.2014.09.005.

7. APPENDICES

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Prereactivation propranolol fails to reduce skin conductance reactivity to prepared fear-conditioned stimuli

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Abstract

Pharmacologic blockade of memory reconsolidation has been demonstrated in fear-conditioned rodents and humans and may provide a means to reduce fearfulness in anxiety disorders and posttraumatic stress disorder. Studying the efficacy of potential interventions in clinical populations is challenging, creating a need for paradigms within which candidate reconsolidation-blocking interventions can be readily tested. We used videos of biologically prepared conditioned stimuli (tarantulas) to test the efficacy of propranolol in blocking reconsolidation of conditioned fear in healthy young adults. Strong differential conditioning, measured by skin conductance, was observed among a screened subset of participants during acquisition. However, subsequent propranolol failed to reduce reactivity to the reactivated conditioned stimulus. These results are consistent with other recent findings and point to a need for testing other candidate drugs.

Descriptors: Fear conditioning, Reconsolidation, Skin conductance, Propranolol, Posttraumatic stress disorder (PTSD)

Once formed, a fear memory must stabilize if it is to persist. This process, termed consolidation, occurs within a narrow window (i.e., minutes to hours), during which the memory is labile and susceptible to intervention (Dudai, 2004; Walker, Brakefield, Hobson, & Stickgold, 2003). Potential clinical opportunities arise from this consolidation window, including interventions for posttraumatic stress disorder (PTSD). Stress hormones may potentiate consolidation and thereby produce a memory trace that is easily activated and resistant to extinction. This process may be involved in the pathogenesis of PTSD (Pitman, 1989). Pharmacological agents, including beta-adrenergic antagonists, could limit the memory-modulating effects of these hormones (McGaugh, 2004) and in so doing attenuate excessive consolidation, if administered during the window. However, this approach is complicated by the need to intervene before the memory has consolidated. Studies attempting to do this have produced mixed results (Hoge et al., 2012; Holmes, James, Coode-Bate, & Deeprose, 2009; Krauseneck et al., 2010; Nugent et al., 2010; Pitman et al., 2002; Stein, Kerridge, Dimsdale, & Hoyt, 2007; Vaiva et al., 2003).

As demonstrated in animal research, reactivation (i.e., retrieval) of a consolidated memory returns it to a destabilized state, from which it must be restabilized (i.e., reconsolidated) if it is to persist (Debiec & Ledoux, 2004; Nader & Einarsson, 2010; Nader, Schafe, & Le Doux, 2000). Reconsolidation is governed by neurobiological processes similar to those of consolidation (Lee, Everitt, & Thomas, 2004), and is also susceptible to pharmacological blockade at the level of stress hormone receptors (Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Pitman et al., 2011; Przybyslawski, Roullet, & Sara, 1999). Because reactivation of trauma memories can be planned in advance, but traumatic events cannot, interference with memory reconsolidation may offer a more feasible clinical target. Brunet and colleagues have extended the above reconsolidation findings to individuals with PTSD (Brunet et al., 2008). Within a double-blind randomized control trial, those participants who received propranolol prior to recalling their traumatic memory exhibited significantly lower overall physiological reactivity during a subsequent laboratory visit when they again recalled their traumatic experience, suggesting that the traumatic memory, or at least its emotional component, had been weakened.

Although clinical application remains the ultimate goal, there persists the need for a basic paradigm wherein candidate pharmacological agents can be more readily tested. Relatively few studies have investigated reconsolidation blockade in humans, and fewer still have done so in a normal (i.e., non-PTSD) population. Recent studies performed in healthy human subjects have helped to address this gap (Kindt, Soeter, & Vervliet, 2009; Soeter & Kindt, 2010, 2011). Soeter and Kindt (2010) used pictures of spiders as a fear-relevant conditioned stimulus (CS) in a differential fear-conditioning paradigm with potentiated eye blink startle response serving as the conditioned response (CR). After first learning to associate one spider (CS+), but not another spider (CS−), with shock, participants received either propranolol or placebo in con-

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Pre-Reactivation Propranolol Fails to Reduce Skin Conductance Reactivity to Fear-Conditioned Stimuli

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Introduction

Reactivation of a consolidated memory may return it to a labile state from which it must be restabilized in order to persist. Blocking this reconsolidation process offers novel avenues for treatment of post-traumatic stress disorder. However, recent efforts in normal humans completely abolished fear responses, thus precluding a comparison of the relative efficacies of the interventions. *We aimed to:*

1. Establish an experimental assay in the form of an optimal Pavlovian differential fear conditioning paradigm, within which to compare the relative strengths of behavioral and pharmacological reconsolidation-based interventions.
2. Determine whether the β-blocker propranolol, administered pre-reactivation, blocks reconsolidation of fear memory as evidenced by reduced sympathetic reactivity.

Methods

• Differential conditioning paradigm utilizes: a) biologically-prepared video stimuli (i.e. tarantulas), and b) sensitive subject population (i.e. healthy, 18-35, afraid of spiders).

• 4 study visits

• *Day 1* (Context A): Conditioning with three tarantula stimuli: CS+, to-be Reactivated (CS+R) and CS+, not to be Reactivated (CS+N) are both paired with shock; CS- is not.

Figure 1: Still images of high-definition video tarantula stimuli. The two labeled "CS+" act as either CS+R or CS+N (randomized by participant). The third is always the CS-. All images taken from a single context.

• *Day 2* (Context B): 40mg propranolol followed 90 minutes later by a single unreinforced reactivation of CS+R.

• *Day 3* (Context A): Tests for renewal and reinstatement, which probe the latent fear memory.

• *Day 30* (Context C): Tests for spontaneous recovery and savings (re-acquisition), which probe the latent fear memory.

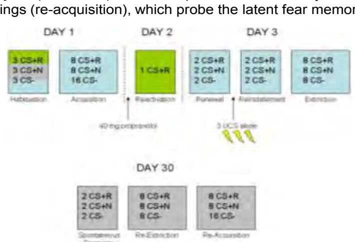

Figure 2: Protocol consists of four visits over one month. Boxes indicate the phase of the conditioning paradigm. Colors represent the three contexts (i.e. A, B, and C) in which stimuli are presented.

Results

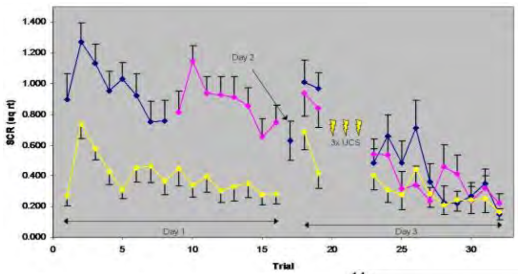

Figure 3: Days 1-3. Mean skin conductance response (μS) by trial across subjects. Shown are participants screened for strong differential conditioning on day 1 (n=19). Blue=CS+R, Pink=CS+N, Yellow=CS-. Standard error bars are included.

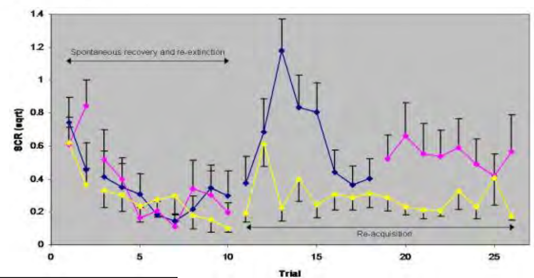

Figure 4: Day 30. Mean skin conductance response (μS) by trial across subjects. Shown are participants screened for strong differential conditioning on day 1 (n=15). Blue = CS+R, Pink = CS+N, Yellow = CS-. Standard error bars are included.

	Statistical Tests	
Day 1	**Acquisition (n=19; 8 trials)**	
	CS+R v CS- : F(1,18) = 66.09**	
	CS+N v CS- : F(1,18) = 38.44**	
	CS+R v CS+N : F(1,18) < 1	
Day 3	**Renewal (n=19; 2 trials)**	**Reinstatement (n=19; 2 trials)**
	CS+R v CS- : F(1,18) = 14.79**	CS+R v CS- : F(1,18) = 6.39*
	CS+N v CS- : F(1,18) = 10.26**	CS+N v CS- : F(1,18) = 4.58*
	CS+R v CS+N : F(1,18) < 1	CS+R v CS+N : F(1,18) < 1
Day 30	**Spont. Recov. (n=15; 2 trials)**	**Savings (n=14; 8 trials)**
	CS+R v CS- : F(1,14) < 1	CS+R v CS- : F(1,13) = 8.69*
	CS+N v CS- : F(1,14) = 6.66*	CS+N v CS- : F(1,13) = 5.21*
	CS+R v CS+N : F(1,14) < 1	CS+R v CS+N : F(1,13) < 1

Table 1: ANOVA main effect of stimulus arranged by test and comparison. Acquisition takes place on day 1, whereas renewal/ reinstatement take place on day 3, and spontaneous recovery/ re-acquisition take place on day 30. Asterisks indicate significance: *<.05, **<.01.

Discussion

• Novel fear-conditioning paradigm elicits strong differential conditioning utilizing biologically prepared stimuli and sensitive subject population.

• Probes for latent fear memory (i.e. renewal, reinstatement, spontaneous recovery, and savings) showed comparable reactivity to CS+R and CS+N.

• 40mg pre-reactivation propranolol failed to significantly reduce conditioned fear *as measured by skin conductance reactivity* to CS+R compared to CS+N. In terms of propranolol, we were unsuccessful in developing a useful experimental assay

• Replicates previous negative results obtained using skin conductance (SC). Fear-potentiated startle (FPS) has yielded positive results, but it wasn't studied here. However, FPS may be less relevant than SC to posttraumatic stress disorder.

References

Kindt M, Soeter M, Vervliet B. Beyond extinction: erasing human fear responses and preventing the return of fear. *Nat Neurosci* 2009;12:256-258.

Schiller D, Monfils MH, Raio CM, Johnson DC, Ledoux JE, Phelps EA. Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature* 2010;463:49-53.

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